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(54) Title: INSECT CHYMOTRYPSIN AND INHIBITORS THEREOF

(57) Abstract: The present invention relates generally to a novel chymotrypsin that exhibits resistance to a plant serine proteinase inhibitor. More particularly, the present invention provides a chymotrypsin which is up-regulated in the gut of *Helicoverpa armigera* and *Helicoverpa punctigera* insect larvae when fed the serine proteinase inhibitors of *Nicotiana alata*. The novel chymotrypsin represents, therefore, a target for the identification of antagonists including inhibitors which are proposed to be useful in the control of *Helicoverpa* spp. populations that have become resistant to serine proteinase inhibitors produced in plants. The antagonists of the chymotrypsin may be topically applied to the plants or, when in proteinaceous form, may be produced by genetic means in plant cells. The antagonists may act at the level of gene expression or protein activity.

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INSECT CHYMOTRYPSIN AND INHIBITORS THEREOF

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a novel chymotrypsin that exhibits resistance to a plant serine proteinase inhibitor. More particularly, the present invention provides a chymotrypsin which is up-regulated in the gut of *Helicoverpa armigera* and *Helicoverpa punctigera* insect larvae when fed the serine proteinase inhibitors of *Nicotiana alata*. The novel chymotrypsin represents, therefore, a target for the identification of antagonists including inhibitors which are proposed to be useful in the control of *Helicoverpa* spp. populations that have become resistant to serine proteinase inhibitors produced in plants. The antagonists of the chymotrypsin may be topically applied to the plants or, when in proteinaceous form, may be produced by genetic means in plant cells. The antagonists may act at the level of gene expression or protein activity.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to in this specification are also collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Female reproductive tissues and wounded leaves of the ornamental tobacco, *Nicotiana alata* amass high levels of serine proteinase inhibitors (PIs) for protection against pests and pathogens (Atkinson *et al., The Plant Cell 5:* 203-213, 1993). These 6 kDa PIs accumulate in the vacuole (Miller *et al., Plant Cell 11:* 1499-1508, 1999) and are derived *in vivo* from the post-translational modification of a 40.3kDa precursor protein. The precursor of the PI

protein (referred to as "NaPI") is composed of six repeated regions of high sequence identity (Figure 1) each with a potential PI reactive site. Processing of the six-repeat precursor protein unexpectedly occurs at sites located within, rather than between the repeated regions. Complete removal of the linker sequence (Glu-Glu-Lys-Lys-Asn) [SEQ ID NO:1] contained within each repeated region, generates five contiguous 6 kDa inhibitors (C1 and T1-T4) and a novel two-chain chymotrypsin inhibitor (C2) formed by disulphide bond linkage of N-terminal and C-terminal peptide fragments (Heath *et al.*, *European Journal of Biochemistry 230(1):* 25-257, 1995; Lee *et al.*, *Nature Structural Biology 6(6):* 526-530, 1999). The structures of C1, T1-T4 and C2 have been solved using ^{1}H-NMR techniques (Nielson *et al.*, *J. Mol. Biol. 242:* 231-243, 1994; Nielson *et al.*, *Biochemistry 34:* 14304-14311, 1995; Lee *et al.*, 1999, *supra*).

Nicotiana alata also has a second gene related to NaPI that encodes a closely related precursor protein with four rather than six repeated domains (Miller *et al.*, *Plant Mol. Biol. 42:* 329-333, 2000). This precursor is also processed *in vivo* resulting in the release of three contiguous 6 kDa inhibitors (C1, T4 and T5) and the two-chain inhibitor C2 (Figure 1). Three of the inhibitors (C1, C2 and T4) are identical to those released from the six-domain precursor. Related multidomain precursors have been described for other solanaceous plants including *N. tabacum* (Balandin *et al.*, *Plant Mol. Biol. 27:* 1197-1204, 1995), *N. glutinosa* (Choi *et al.*, *Biochim. et Biophys. Acta 1492:* 211-215, 2000), *L. esculentum* (Taylor *et al.*, *Plant Mol. Biol. 23:* 1005-1014, 1993) and *Capsicum annum* (Moura and Ryan, *Plant Physiol. 126:* 289-298, 2001; Antcheva *et al.*, *Protein Sci. 10:* 2280-2290, 2001).

Several groups have reported on the affect of serine proteinase inhibitors on the activity of the digestive proteases of insects and have suggested that they are produced by plants for protection against the damaging affects of insect pests and microorganisms (Ryan, *Annu. Rev. Phytopathol. 28:* 425-449, 1990; Gatehouse *et al.*, In: *Plant Genetic Manipulation for Crop Protection*, Biotech. in Agriculture No. 7, Eds. Gatehouse, Hilder & Boulter, International U.K., pp. 155-181, 1992). Insects that are specialist feeders on a particular host plant are generally resistant to the serine PIs produced by that plant, but are sensitive

to PIs produced by non-hosts (Broadway and Villani, *Entomol. Expo. Appl. 76:* 303-312, 1995; Broadway, *J. Insect. Physiol. 41:* 107-116, 1995). There is interest, therefore, in transferring genes encoding serine PIs from non-hosts into crop plants to enhance insect resistance and to decrease reliance on chemical pesticides. Recently, however, several groups have reported on the ability of certain insects to change the relative proportions of proteolytic enzymes in their midgut following ingestion of high levels of PIs (Broadway, 1995, *supra*; Jongsma *et al., Proc. Natl. Acad. Sci. USA 92(17):* 8041-8045, 1995a). Broadway (1995, *supra*), for example, found that certain lepidopteran insects produce two broad classes of trypsin like proteases, one of which is insensitive to PIs from cabbage leaves. After ingestion of the cabbage PIs the insects increased production of the trypsin class not affected by the PIs and thus were able to grow and develop unhindered. Jongsma and coworkers (1995, *supra*) made a similar observation with *Spodoptera exigua* larvae fed on PIs from potato (PotII) and tobacco. The factors that regulate the secretion of these proteases under these conditions are not known.

These studies indicate that PIs specific for only one or two of the range of proteinases in an insect gut will be of limited use for long term plant protection. The gene encoding the *N.alata* PI has a potential advantage over other plant PIs for the enhancement of insect resistance in transgenic plants. Most plant serine PIs contain only one or two inhibitory domains, whereas the *N. alata* PI precursors have four or six (Figure 1). Thus, there is potential to engineer the individual domains of the *N. alata* PI to provide inhibitory activity against several proteinases in the insect gut.

The midgut proteases of several *Lepidoptera*, *Coleoptera* and *Orthoptera* have been partially characterized. In most Lepidopteran species the endoproteinase activity is due primarily to serine proteinases (trypsin, chymotrypsin and/or elastase) and cysteine and metalloproteinases are not detectable (Christeller *et al., Insect Biochem. Molecul. Biol. 22:* 735-746, 1992; Terra and Ferreira, *Comp. Biochem. Physiol. 109:* 1-62, 1994; Xu and Qin, *J. Econ. Entomol. 87:* 334-338, 1994; Lee and Anstee, *Insect. Biochem. Molec. Biol. 25:* 63-71, 1995a; Johnston *et al., Insect Biochem. 21:* 389-397, 1991; Johnston *et al., Insect Biochem. Molec. Biol. 25(3):* 375-383, 1995). Exopeptidase and leucineaminopeptidase

have also been identified (Christeller *et al.*, 1992, *supra*; Lee and Anstee, *Insect. Biochem. Molec. Biol. 25(1):* 49-61, 1995b).

The mechanism of action of PIs on insects is only partially understood. Three responses have been described:-

(i) *Severe retardation of growth without a decrease in gut proteolytic activity.* Broadway and Duffey (*J. Insect Physiol. 32:* 673-680, 1986a; Broadway and Duffey, *J. Insect Physiol. 32:* 827-833, 1986b) found that insects fed on PIs had remarkably reduced growth rates that were not associated with a decrease in the total proteolytic activity in the gut. Indeed the gut proteolytic activity often increased. They suggested that a feedback mechanism was operating that led to hyperproduction of proteases, that led in turn to a depletion of essential sulphur containing amino acids. This phenomenon has been recorded for other insects after chronic ingestion of PIs (Burgess *et al., Entomol. Exp. App. 61:* 123-130, 1991; De Leo *et al., Plant Physiol. 118:* 997-1004, 1998; Markwick *et al., J. Economic Entomology 91* (6*):* 1265-76, 1998).

(ii) *Severe retardation of growth with a decrease in gut proteolytic activity.* Broadway (1995, *supra*) found that the lepidopteran species, *Agrotis ipsilon* (black cutworm) had reduced growth and delayed pupation after exposure to soybean trypsin inhibitor and did not respond by secreting PI-insensitive proteases. These insects had up to a 70% reduction in total gut proteolytic activity. Codling moth larvae (*Lepidoptera*:Tortricidae) fed on 'elastase inhibitors' were also retarded in growth and development that was associated with diminished elastase activity in the gut (Markwick *et al., Journal of Economic Entomology 88(1):* 33-39, 1995).

(iii) *No effect on growth - change in the complement of gut proteinases.* Some insects can compensate for the inhibition of one group of proteinases by inducing a new proteinase activity. The genomes of lepidopteran insects contain genes for a range of serine proteases and insects can modify the expression of specific isozymes to

suit dietary components (Bown, *et al., Insect Biochem. Molec. Biol. 27:* 625-638, 1997; Broadway, *J. Insect. Physiol. 43(9):* 855-874, 1997). Changes in the complement of gut trypsins and chymotrypsins have been detected using Northern blot analysis on RNA from *H. armigera* (Bown, *et al.*, 1997, *supra*; Gatehouse, *et al., Insect Biochem. Molecul. Biol. 27:* 929-944, 1997), *H. zea* and *Agrotis ipsilon* (Mazumdar-Leighton and Broadway, *Insect Biochem. Mol. Biol. 31:* 645-657, 2001a; Mazumdar-Leighton and Broadway, *Insect Biochem. Mol. Biol. 31:*633-644, 2001b). Corresponding changes at the protein level have also been observed using electrophoretic separation of isozymes for *H. armigera* (Harsulkar, *et al., Plant Physiol. 121:* 497-506, 1999; Patankar, *et al., Insect Biochem. & Mol. Biol. 31:* 453-464, 2001), *Spodoptera frugiperda* (Paulillo, *et al., J. Econ. Entomol. 93:*892-896, 2000), *H. zea* and *Trichoplusia ni* (Broadway, *Arch. Insect Biochem. Physiol. 32(1):* 39-53, 1996). Sometimes specific isozymes have been up-regulated, and occasionally proteases previously undetected have been observed.

Recently, Mazumdar-Leighton and Broadway (2001a, *supra*) demonstrated that the production of PI-insensitive trypsins in *H. zea* is regulated at the transcriptional level and can be abolished using the transcriptional regulator actinomycin. Broadway and colleagues examined changes in gut trypsin and chymotrypsin activity after *H. zea* and *Trichoplusia ni* larvae were fed for 48 h on artificial diet containing 1% SBTI (Broadway, 1996, *supra*). Trypsin activity increased after SBTI consumption and protease banding patterns on zymograms indicated a change in the relative complement of proteases. The researchers showed *in vitro* that SBTI could inhibit 74% of the trypsin activity in gut extracts from control larvae, but only 3% of the gut trypsin activity in larvae that had consumed SBTI. They suggested the new protease bands (one new band for *H. zea* and 6 new bands for *T. ni*) on the zymograms may be SBTI-insensitive trypsins or SBTI-insensitive chymotrypsins and concluded that the production of these new proteases was enhanced by the ingestion of SBTI. Further studies using Northern blot analysis showed that consumption of SBTI resulted in transcriptional induction of mRNAs encoding trypsins and chymotrypsins by *H. zea* and *Agrotis ipsilon* (Mazumdar-Leighton and Broadway, 2001a, *supra*; Mazumdar-Leighton and Broadway, 2001b, *supra*), although it was not

determined if these proteases were SBTI-insensitive.

The novel trypsin transcript induced in *H. zea* after ingestion of SBTI was designated HzT15 (Mazumdar-Leighton and Broadway, 2001b, *supra*). Recently, the first insect digestive enzyme insensitive to several proteinase inhibitors was purified from the gut of *H. zea* and corresponds to the protein encoded by HzT15 (Volpicella *et al.*, *Eur. J. Biochem. 270:* 10-19, 2003). The authors identified several differences in charge distribution across the surface of the structural model of this PI-insensitive trypsin relative to the PI inhibitable trypsins, but were unable to identify the structural changes that led to resistance.

Until recently, chymotrypsins were assumed to contribute relatively little to protein digestion in *Lepidoptera* and consequently most biochemical studies focused on characterization of the trypsins. This problem arose due to the initial use of synthetic substrates that worked well with mammalian chymotrypsins, but not at all or poorly with the Lepidopteran enzymes. Lepidopteran chymotrypsins prefer synthetic substrates with at least four amino acids to occupy the S1-S4 binding subsites on the enzyme, whereas mammalian trypsins are active on shorter substrates with one amino acid that is specific for the S1 binding subsite. That is, the insect chymotrypsins appear to have an extended substrate binding site requiring at least four amino acids for efficient catalysis. Recent studies have shown that chymotrypsins do respond to PI ingestion and are worthy of more detailed investigation. When larvae from *H. armigera* were fed on diets consisting of either potato proteinase inhibitor II, soybean trypsin inhibitor, aprotin (trypsin inhibitor) or potato proteinase inhibitor I, levels of chymotrypsin mRNA increased in all cases while trypsin mRNA decreased (Gatehouse *et al.*, 1997, *supra*). Other reports also mention upregulation of chymotrypsins in preference to trypsins (Bown *et al.*, 1997, *supra*; Wu *et al.*, *Molecular Breeding 3:* 371-380, 1997). Mazumdar-Leighton and Broadway (2001a, *supra*) assayed chymotrypsin activity in the gut of *H. zea* larvae and found that SBTI inhibited 95% of the chymotrypsin from the gut of control insects but only 35% of activity from the gut of insects that had prior exposure to SBTI in the diet.

Hence, consumption of proteinase inhibitors can lead to a drastic change in the complement of gut proteases which allows insects to adapt to the diet and survive. Changes in the complement of proteases after exposure to PIs have been detected in insects fed on both artificial diets and transgenic plants. The triggers that regulate these changes are still unknown and the responses vary with the species, the PI and its concentration, and the base diet. It is unclear why some inhibitors induce this response and others do not. It is clear, however, that some larvae are genetically pre-adapted to PIs, since prior exposure to a specific inhibitor is not necessary for an insect to be resistant (Broadway, 1996, *supra*).

There is a need to identify and investigate novel insect proteinases which are insensitive to PIs and to use these to screen for antagonists of the proteinases in order to develop agents useful in controlling insect growth, maintenance, development and/or survival.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided at the end of the specification.

The present invention provides a novel chymotrypsin from *Helicoverpa* spp. referred to herein as "HpCh5". Reference to "HpCh5" includes all variants, derivatives, homologs and analogs as well as members of a HpCh5 family of chymotrypsins. Examples of variants of HpCh5 include proteinase inhibitor (PI) sensitive forms. Such sensitive forms may carry *inter alia* a substitution of the arginine at position 192 to an asparagine or glutamine. This substitution is referred to herein as "R192N/Q" using single amino acid nomenclature or "Arg 192 Asn/Gln" using three letter amino acid code. Other derivatives of HpF5 include the signal sequence of HpF5.

The HpCh5 chymotrypsin is encoded by a nucleotide sequence referred to as "HpF5". Again, reference to "HpF5" includes variants, homologs and analogs thereof. The term "HpF5" encompasses both a genomic sequence as well as a cDNA sequence. The amino acid sequence of HpCh5 is set forth in SEQ ID NO:2. The amino acid sequence of the N-terminal activation peptide is shown in SEQ ID NO:3. The nucleotide sequence of the coding region of HpF5 is set forth in SEQ ID NO:4 with the nucleotide sequence encoding the activation peptide is shown in SEQ ID NO:5 and its entire 5'-3' sequence shown in SEQ ID NO:6. HpCh5 is generally characterized by being substantially insensitive to inhibition by a PI from *N. alata*.

Variants and homologs of HpCh5 include molecules having at least 75% amino acid identity to SEQ ID NO:2 after optimal alignment. Variants and homologs of HpF5 include nucleotide sequences having at least about 75% similarity to SEQ ID NO:4 or SEQ ID NO:6 after optimal alignment or a nucleotide sequence capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding a chymotrypsin from *Helicoverpa ssp.* or a variant, derivative, homolog or analog of said chymotrypsin, wherein said chymotrypsin exhibits resistance to a PI from *N. alata.*

Another aspect of the present invention provides an isolated chymotrypsin from *Helicoverpa* ssp. wherein said chymotrypsin exhibits resistance to a PI from *N. alata* or a variant, derivative, homolog or analog of said chymotrypsin.

The present invention provides compounds which inhibit HpCh5 or its variants and homologs or members of the HpCh5 chymotrypsin family or which inhibit expression of the HpF5 gene or its variants and homologs.

The compounds may be chemical type compounds such as those sprayed or provided to plants or genetic type molecules which may be either topically applied or generated in plant cells. The HpCh5 or HpF5 antagonists may also be a modified form of an existing plant PI.

The present invention provides, therefore, methods for inhibiting insect infestation of a plant or for retarding insect growth and development by the application or dispersement of an antagonist of HpCh5 activity or HpF5 gene expression.

The antagonists include compounds which bind to and inhibit HpCh5 as well as antisense or sense nucleic acid molecules generated by a plant cell and then ingested by an insect.

Reference to an "antagonist" includes reference to an inhibitor.

The present invention further provides genetically modified plants which are engineered to produce a HpCh5 or HpF5 antagonist. Reference to a "plant" includes a monocotyledonous or dicotyledonous plant and may be a plant regenerated from genetically transformed callus or tissue or progeny of such a plant. The present invention further provides seeds and other reproductive material from the genetically modified plants of the present invention.

Plants contemplated herein include cotton, sweet corn, tomato, tobacco, piniento, potato, sunflower, citrus, plums, sorghum, leeks, soybean, alfalfa, beans, pidgeon peas, chick peas, artichokes, curcurbits, lettuce, *Dianthus* (an ornamental plant) and geraniums, cape gooseberry, maize, flax and linseed, alfalfa, lupins, broad beans, garden peas, peanuts, canola, snapdragons, cherry, sunflower, pot marigolds, *Helichrysum* (an ornamental plant), wheat, barley, oats, triticale, carrots, onions orchids, roses and/or petunias.

The present invention further provides nucleic acid molecules which encode potato-derived protenase inhibitors such as but not limited to Pot1A and Pot1B or their homologs or derivatives as well as transgenic plants comprising and capable of expressing same.

Yet another aspect of the present invention contemplates a method for screening for an inhibitor of an insect chymotrypsin which is insensitive to inhibition by NaPI such as HpCh5. Such a method generally involves testing for chymotryptic activity in the presence of potential inhibitors. The assay is conveniently contacted *in vitro* although the use of *H. argmigera* and/or *H. punctiga* is also encompassed by the present invention. An isolated inhibitor identified by the subject assay is also contemplated by the present invention.

A summary of sequence identifiers used throughout the subject specification is provided in Table 1.

TABLE 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	linker sequence
2	Amino acid sequence of HpCh5 [Figure 12]
3	Amino acid sequence of the activation peptide of HpCh5 [Figure 12]
4	Nucleotide sequence of coding region of mature chymotrypsin domain of HpF5 [Figure 12]
5	Nucleotide sequence of activation peptide of HpF5 [Figure 12]
6	Nucleotide sequence encoding activation peptide and HpCh5 mature chymotrypsin domain together with 3' UTR [Figure 12]
7	*Bam*HI oligonucleotide primer
8	*Hin*dIII oligonucleotide primer
9	N-terminal sequence of NaPI-insensitive chymotrypsin HpCh5 [Table 7, Figure 11B]
10	*Fw2ResChy* primer [Table 7, Figure 11B]
11	FwResChym primer [Table 7, Figure 11B]
12	Hc35PQE-60-Fw primer
13	Hc35PQE-60-Rv primer
14	gene specific sense primer
15	gene specific antisense primer
16	StPotIA sense primer
17	StPotIB sense primer
18	StPotIA/B antisense primer
19	FWBacRECH1 (5'-3') primer
20	FWBacRECH2 (5'-3') primer
21	RvRECH (3'-5') primer

SEQUENCE ID NO:	DESCRIPTION
22	N-terminal amino acid sequence of six domain PI precursor from *N. alata* [Figure 1C]
23	Amino acid sequence of C1 peptide from six domain PI precursor from *N. alata* [Figure 1C]
24	Amino acid sequence of T1 peptide from six domain PI precursor from *N. alata* [Figure 1C]
25	Amino acid sequence of T2 and T3 peptides from six domain PI precursor from *N. alata* [Figure 1C]
26	Amino acid sequence of T4 peptide from six domain PI precursor from *N. alata* [Figure 1C]
27	C-terminal amino acid sequence of six domain PI precursor from *N. alata* [Figure 1C]
28-31	Amino acid sequence of peptide fragment of *Helicoverpa punctigera* chymotrypsin [Figure 5B]
32	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72966) [Figure 7]
33	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72959) [Figure 7]
34	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72960) [Figure 7]
35	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72958) [Figure 7]
36	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72952) [Figure 7]
37	Amino acid sequence of chymotrypsin from *H. armigera* (CAA72951) [Figure 7]
38	FWG1 primer [Figure 8]
39	RVG4 primer [Figure 8]
40	Y79Fw primer [Figure 8]
41	Y72Fw primer [Figure 8]
42	Y72Rv primer [Figure 8]
43	Amino acid sequence of *H. punctigera* chymotrypsin (F1Apcr) [Figure 9]
44	Amino acid sequence of *H. punctigera* chymotrypsin (F1Bpcr) [Figure 9]
45	Amino acid sequence of *H. punctigera* chymotrypsin (F2Bpcr) [Figure 9]
46	Amino acid sequence of *H. punctigera* chymotrypsin (F3pcr) [Figure 9]
47	Amino acid sequence of *H. punctigera* chymotrypsin (F4pcr) [Figure 9]

SEQUENCE ID NO:	DESCRIPTION
48	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh1AI) [Figure 10]
49	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh1BI) [Figure 10]
50	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh2A) [Figure 10]
51	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh2B) [Figure 10]
52	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh3A) [Figure 10]
53	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh4I) [Figure 10]
54	Amino acid sequence of chymotrypsin from *H. punctigera* (HpCh4II) [Figure 10]
55	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Rech1a) [Figure 11A]
56	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Rech1b) [Figure 11A]
57	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family1a) [Figure 11A]
58	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family1b) [Figure 11A]
59	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family2b) [Figure 11A]
60	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family2a) [Figure 11A]
61	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family3) [Figure 11A]
62	Amino acid sequence of peptide from *H. punctigera* chymotrypsin (Family4) [Figure 11A]
63	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh1AI) [Figure 13]
64	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh1BI) [Figure 13]
65	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh2B) [Figure 13]
66	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh2A) [Figure 13]
67	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh3) [Figure 13]
68	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh4I) [Figure 13]
69	Amino acid sequence of *H. punctigera* chymotrypsin (HpCh5) [Figure 13]

SEQUENCE ID NO:	DESCRIPTION
70	Amino acid sequence of human brain trypsin (TrypsinIV) [Figure 13]
71	Amino acid sequence of chymotrypsin from *H. armigera* [Figure 14]
72	Amino acid sequence of chymotrypsin from *H. punctigera* [Figure 14]
73	Amino acid sequence of bovine chymotrypsin B (BOV CHB) [Figure 15]
74	Amino acid sequence of bovine chymotrypsin A (BOV CHA) [Figure 15]
75	Amino acid sequence from *H. punctigera* (HpCh2A) [Figure 15]
76	Amino acid sequence from *H. punctigera* (HpCh5) [Figure 15]
77	Amino acid sequence of potato inhibitor I family (PotI) {StPotIB} [Figure 24]
78	Amino acid sequence of potato inhibitor I family (PotI) {X67950} [Figure 24]
79	Amino acid sequence of potato inhibitor I family (PotI) {R01052} [Figure 24]
80	Amino acid sequence of potato inhibitor I family (PotI) {M17108} [Figure 24]
81	Amino acid sequence of potato inhibitor I family (PotI) {StPotIA} [Figure 24]
82	Amino acid sequence of potato inhibitor I family (PotI) {K03290} [Figure 24]
83	Amino acid sequence of potato inhibitor I family (PotI) {Z12619} [Figure 24]
84	Amino acid sequence of potato inhibitor I family (PotI) {X78988} [Figure 24]
85	Amino acid sequence of potato inhibitor I family (PotI) {EILXCH} [Figure 24]
86	Nucleotide sequence encoding endoplasmic reticulum peptide [Figure 28]
87	Amino acid sequence of endoplasmic reticulum peptide [Figure 28]
88	Nucleotide sequence of FwBacRECH1 primer [Figure 28]
89	Nucleotide sequence of FwBacRECH2 primer [Figure 28]
90	Nucleotide sequence of HpF5 to which DNA encoding endoplasmic reticulum signal is to be added [Figure 28]
91	Amino acid sequence of HpCh5 to which endoplasmic reticulum signal is to be added [Figure 28]

SEQUENCE ID NO:	DESCRIPTION
92	Nucleotide sequence of RvRECH primer [Figure 28]
93	Amino acid sequence of HpCHY1 [Figure 4C]

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation showing temperature stability of baculovirus expressed *H. punctigera* chymotrypsin. Bovine chymotrypsin (♦) and HpF5 (▲) in 50 mM Na acetate were compared by incubating 100 µL aliquots at 5°C intervals between 40°C and 70°C. After chilling the heated samples on ice, duplicate activity assays were performed by incubating 10 µL of bc or 50 µL of HpF5 with substrate at 30°C. Residual activity was presented as a percentage of the activity of the untreated control.

Figure 2 is a graphical representation showing growth of *H. punctigera* larvae fed on either low protein haricot bean artificial diet or cotton leaf artificial diet, in the presence or absence of 0.26% (w/v) NaPIs. Weight gain (mg) was monitored for 21 days. Larvae grew at a similar rate on both artificial diets and growth was retarded in the presence of NaPIs. **(A)** Growth of larvae. **(B)** Relative size of larvae at 21 days after feeding on cotton artificial diet, in the presence or absence of 0.26% NaPIs. Only five of the 20 larvae fed on NaPI survived and all five weighed less than control larvae after 21 days. **(C)** The effect of NaPIs on gut trypsin and chymotrypsin activity. When each *H. punctigera* larva reached the late fourth/ early fifth instar stage of development, the gut was removed and gut extract prepared prior to assays for trypsin and chymotrypsin activity. Extracts from individual larvae fed on control cotton-leaf artificial diet (CC) are indicated in grey and larvae fed on the same diet containing 0.26% ((w/v)) NaPI (NC) are indicated in black. Only four test larvae survived to fifth instar, whereas most control larvae survived. All assays were performed in duplicate. Trypsin activity was determined using BApNA substrate and chymotrypsin activity with SAAPFpNA substrate. Units of activity are expressed as change in absorbance at 405 nm/min/mg gut extract protein (± standard deviation). Trypsin activity was almost abolished, relative to controls, in extracts from NaPI-fed larvae, while chymotrypsin activity was low in two of the four NaPI-fed larvae. **(D)** The effect of NaPIs on trypsin and chymotrypsin activity in the frass. Frass was collected from each larva fed on the cotton leaf artificial diet and faecal extracts were prepared. Trypsin and chymotrypsin activity in extracts from larvae fed on the control diet (CC) are indicated in grey and larvae fed on the same diet containing 0.26% ((w/v)) NaPI (NC) are indicated in

black. Note that frass of larvae CC16 and NC13 were included in this analysis but excluded from Figure 2C because the gut were damaged during preparation of extracts. All assays were performed in duplicate. Trypsin activity was determined using BApNA substrate and chymotrypsin activity with SAAPFpNA substrate. Units of activity are expressed as change in absorbance at 405 nm/min/mg faecal extract protein (± standard deviation). Negligible trypsin activity was evident in extracts from NaPI-fed larvae, while chymotrypsin activity was elevated, relative to controls. **(E)** Levels of trypsin in the frass of control and NaPI fed larvae. Frass extracts were analysed on 15% ((w/v)) SDS-PAGE and transferred to nitrocellulose. The nitrocellulose filter was probed with rabbit anti-*Hp*TRY1 serum (1 in 2000 dilution) as the primary antibody, followed by the secondary antibody, donkey anti-rabbit IgG-horse radish peroxidase conjugate (1 in 2000 dilution). Immuno-reactive proteins were visualized using Enhanced Chemiluminescence (ECL) reagents and HyperfilmECL X-ray film. The trypsin in the frass of larvae that consumed NaPIs (NC) was significantly increased relative to controls (upper panel) but inactive (lower panel), presumably because it was in complex with the NaPIs. In comparison, trypsin was active in the frass of the control larvae (CC) even though the amount present was below the detection level of the antibody. **(F)** Production of NaPI-insensitive proteases in the gut of NaPI fed larvae. The gut extracts of the larvae were subjected to inhibition assays to identity NaPI-insensitive trypsins and chymotrypsins. Each extract was pre-incubated for 30 min at 30°C in the presence or absence of 80 nM NaPI inhibitor (T1 monomer for trypsin assays and C1 monomer for chymotrypsin assays), prior to the addition of substrate to initiate the reaction. Results from extracts assayed without inhibitor are indicated in grey(control larvae) and black (NaPI-fed larvae). Results from extracts assayed with inhibitor are stippled. All assays were performed in duplicate. Units of activity are expressed as substrate hydrolysis at 405 nm/min/mg extract protein (± standard deviation). (A) Inhibition of trypsin activity by T1. Trypsin activity was determined using BApNA substrate. (B) Inhibition of chymotrypsin activity by C1. Chymotrypsin activity was determined with SAAPFpNA substrate. T1 almost totally inhibited trypsin activity in the extracts of control larvae, indicating these larvae did not produce NaPI-insensitive trypsins. The extracts from NaPI-fed larvae contained negligible trypsin activity, but this activity could not be inhibited by T1. C1 did not inhibit chymotrypsin activity in the

extracts of six control larvae and only partially inhibited the gut chymotrypsins in the rest of the controls indicating the control insects contained a C1-insensitive chymotrypsin. Most of the chymotrypsin activity in extracts from NaPI-fed larvae can be attributed to NaPI-insensitive chymotrypsins. When extracts from control and NaPI-fed larvae were pre-incubated with 80 nM chymostatin, all chymotrypsin activity was abolished.

Figure 3 is a graphical representation showing the effect of various proteinase inhibitors on the chymotrypsin activity in unfractionated gut extracts from *H. punctigera*. Proteinase inhibitors were mixed with 1 μg of protein from an unfractionated gut extract before incubation with the chymotrypsin substrate N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Inhibition is expressed as a percentage of the total activity in the control samples.

Figure 4 is a photographic representation showing purification and N-terminal sequence of an NaPI inhibitable chymotrypsin from *H. punctigera* gut. **(A and B)** Protein gel analysis of fractions at various stages of purification using an affinity column with immobilized C1 inhibitor (Figure 1). **(A)** 15% ((w/v)) SDS-polyacrylamide gel loaded with (a) unfractionated gut extract (b) and (c) unbound proteins. **(B)** 12.5% (w/v) SDS-polyacrylamide gel loaded with (d) wash fraction (e) protein bound to the C1 column. **(C)** N-terminal sequence of the ~24 kDa protein in lane (e).

Figure 5 is a diagrammatic representation showing purification and N-terminal sequence of an NaPI-insensitive chymotrypsin from *H. punctigera*. **(A)** PVDF blot of chymotrypsin (i) eluted from potato inhibitor column. Potato Inhibitor II (ii) and potato inhibitor I (iii) both co-eluted from the matrix under denaturing conditions. **(B)** N-terminal amino acid sequence obtained from PVDF blot. Rech1a was the most abundant of the four sequences obtained.

Figure 6 is a graphical representation showing the effect of pH and a range of substrates on the activity of the NaPI-insensitive chymotrypsin from *H. punctigera* midgut. SA_2PF-pNA, N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; SA_2PL-pNA, N-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide; MA_2PM-pNA, N-methoxysuccinyl-Ala-Ala-Pro-Met-*p*-nitroanilide.

Figure 7 is a diagrammatic representation showing the design of oligonucleotide primers for amplification of chymotrypsins from *H. punctigera*. An alignment of chymotrypsins from *Helicoverpa armigera* predicted from DNA sequences in the GenBank database. NCBI protein database accession numbers are left of the sequences. Regions corresponding to the oligonucleotide primers are boxed and the direction of amplification are indicated by arrows.

Figure 8 is a diagrammatic representation showing oligonucleotide sequences used in RT-PCR amplification of *Helicoverpa* chymotrypsins.

Figure 9 is a diagrammatic representation showing PCR products from amplification of cDNAs encoding *H. punctigera* chymotrypsins. PCR amplification of cDNA prepared from gut mRNA yielded partial sequence for five distinct chymotrypsins. The translated sequence is aligned and the region corresponding to the PCR primers is boxed.

Figure 10 is a diagrammatic representation showing alignment of predicted amino acid sequence of chymotrypsins from *H. punctigera*. The catalytic residues are marked by a solid triangle (▼). The highly conserved active site motifs are highlighted with grey. The dipeptide R-I (↓↓), conserved among all chymotrypsins is the site for the proposed cleavage of the activation peptide by trypsin. The residues that lie in the substrate binding pocket and confer substrate specificity are indicated by the symbols ¥,§,#. The cysteine (●) residues are highly conserved among all chymotrypsins.

Figure 11 is a diagrammatic representation showing design of oligonucleotide primers for amplification of cDNA encoding the NaPI-insensitive chymotrypsins from *H. punctigera*. **(A)** Comparison of the N-terminal sequence of two NaPI-insensitive chymotrypsins with *Helicoverpa* chymotrypsins predicted from the cDNA clones. The unique regions F1 and F2 are shaded. **(B)** Oligonucleotide primers complementary to unique regions at the N-terminus of the insensitive chymotrypsin.

Figure 12 is a diagrammatic representation showing nucleotide sequence and deduced amino acid sequence from the cDNA encoding the insensitive chymotrypsin. The nucleotide sequence of the insensitive chymotrypsin cDNA and deduced amino acid sequence. The amino acid sequence obtained from N-terminal sequence of purified protein is shaded in grey. The putative site for endoproteolytic cleavage by trypsin is shown by the arrow. The double underlined regions in the nucleotide sequence refer to the positions of the degenerate primers used for PCR amplification. The polyadenylation signal sequence is single underlined and an asterisk marks the stop codon. The deduced amino acid sequence of the putative activation peptide is numbered -40 to -1 followed by the mature domain (+1). The three amino acids that correspond to the catalytic residues are marked by the symbol #. The chymotrypsin substrate specificity residue, serine, located at the base of the primary substrate-binding pocket is marked with the symbol §.

Figure 13 is a representation showing alignment of *H. punctigera* chymotrypsin families showing sequence identity. ClustalW alignment of members from the five families of *H. punctigera* chymotrypsins. Protein sequence is given in single letter code. Identical amino acids are coloured black, similar amino acids are grey. Amino acids are numbered on the right and gaps have been introduced to maximize the alignment. The NaPI- insensitive chymotrypsins are members of family 5 and are characterized by a unique arginine residue (arrowed) at position 185. Human trypsin IV also contains an arginine residue (arrowed) in a similar position.

Figure 14 is a representation showing alignment of the *H. punctigera* NaPI-insensitive chymotrypsin with a homolog from *H. armigera* that also has an arginine residue at position 185.

Figure 15 is a representation showing the deduced protein sequences for the insensitive (HpCh5) and sensitive (HpCh2A) chymotrypsins from *H. punctigera* aligned to the bovine chymotrypsin isoforms A and B. *H. punctigera* chymotrypsins HpCh2A and HpCh5 were aligned to the bovine chymotrypsin isoforms A and B using ClustalW. The numbering system (excluding gaps) is according to the nomenclature of Greer, *Proteins 7:* 317-34,

1990 used for bovine chymotrypsin. Dots throughout the sequences represent conserved residues. The regions shaded in grey designate residues that form surface loops that are involved in recognition and binding of substrates or inhibitors. The primary substrate-binding pocket is formed by the regions labeled S1A, S1B and S1C. The S1' site is formed by loops 35 and 60. Black boxes mark residues in the HpF5 sequence that differ significantly to amino acids in the corresponding positions in other chymotrypsins.Using the Greer, 1990, *supra* nomenclature these residues are Asp36, Arg 63, Thr72, Pro 83, Gly 109, Ileu 120, Glu insertion between 129 and 130, Glu 134, Ser145, Arg 192 and Pro 207. The boxed amino acids are removed from bovine chymotrypsins by autocatalytic cleavage that results in the formation of α-chymotrypsin.

Figure 16 is a diagrammatic representation showing several surface loops in the structural model of *H. punctigera* chymotrypsin HpCh2A are larger than the cognate loops in bovine chymotrypsin. The structural model of *H. punctigera* F2A chymotrypsin (grey) was superimposed onto the structure of alpha-chymotrypsin from *Bos taurus* (black). Surface loops 60, 35, and 142 that are implicated in substrate recognition are larger in the insect chymotrypsin model. The chymotrypsin substrate specificity residue, serine 189, positioned at the base of the primary substrate-binding pocket is viewed as a space filled representation of the van der Waals radius.

Figure 17 is a representation showing glutamine 192 (Greer, *1990, supra* nomenclature, Figure 15) of the sensitive chymotrypsin HpCh2A appears easily accommodated when modeled in complex with C1. Structural model of the sensitive chymotrypsin HpCh2A (grey) in complex with the proteinase inhibitor C1 (black). The side chain of glutamine 192 is arrowed The residues of C1 in the vicinity of Gln 192 are represented in stick configuration (black).

Figure 18 is a diagrammatic representation showing comparison of the environment surrounding residue 192 of the sensitive and insensitive chymotrypsin complexed to C1. Enlarged view of the boxed area shown in Figure 17. Arginine 192 of the insensitive

chymotrypsin (B) appears to clash with residues of the C1 inhibitor, in contrast there is no apparent conflict with glutamine 192 of the sensitive chymotrypsin (A).

Figure 19 is a diagrammatic representation showing the environment surrounding arginine 192 when the insensitive chymotrypsin is complexed to the Type 1 potato proteinase inhibitor (PotIB, Fig24). **(A)** Structural model of the insensitive chymotrypsin HpCh5 (grey) in complex with the potato type I proteinase inhibitor PotI (black). **(B)** Enlarged view of the region around Arg 192 (boxed area in B). The side chain of arginine 192 is labeled. The residues of PotI in the vicinity of Arg 192 are represented in stick configuration (black).

Figure 20 is a photographic representation showing expression of the chymotrypsin clone HpF2B in *E. coli* cells for the production of a polyclonal antibody. **(A)** The separation of total cell lysates taken at time points 0-5 hr after induction of HpCh2B on a 12.5% (w/v) SDS-PAGE gel stained with Coomassie Blue. The lanes are marked by the number of hours after induction and the arrow indicates the position of the induced protein with the correct predicted molecular mass. **(B)** *Panel 1:* Bacterially expressed HpCh2B purified on Talon resin (BD Biosciences Clontech), separated on a 15% (w/v) SDS-PAGE gel and stained with Coomassie Blue. *Panel 2:* Identical sample to Panel 1 transferred to nitrocellulose and immunostained with anti-HpCh2B antibodies. **(C)** Decreasing amounts (200, 150, 100, 75, 50, 25, 20, 10, 0 ng) of bacterially expressed chymotrypsin HpCh2B separated by SDS-PAGE and stained with silver and a protein blot of an identical gel probed with anti-chymotrypsin HpCh2B antibodies (1/2500). The *H. punctigera* antibody had a detection limit of 20 ng of bacterially expressed protein.

Figure 21 is a photographic representation showing specificity of antibodies raised against bacterially expressed NaPI-insensitive (HpCh5) and sensitive (HpCh2B) chymotrypsins from *H. punctigera*. Bacterially expressed NaPI-insensitive (R) and sensitive (C) chymotrypsins were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels and **(A)** stained with Coomassie Blue, **(B)** immunoblotted with an α-His tag antibody, **(C)**

immunoblotted with the antibody to the HpCh2B chymotrypsin (α-RC), and **(D)** immunoblotted with the antibody to the HpCh5 chymotrypsin (α-SC).

Figure 22 are representations showing purification of PotI from potato tubers. PotI was purified from potato tubers (Russet Burbank) by acid extraction, ammonium sulphate precipitation and gel filtration. **(A)** SDS-PAGE stained with silver. lane 1: molecular size markers (kDa), lane 2: pooled PotI containing fractions from G-75 column, lane 3: immunoblot of lane 2 using an antibody raised in rabbits to a commercial preparation of PotI (Calbiochem) linked to keyhole limpet hemocyanin. PotI was identified as a single band with an approximate mass of 6 kDa. **(B)** RP-HPLC of pooled G-75 fractions from A. Peaks 1, 2 and 3 are PotI isoforms, peak 4 is a contaminating protein.

Figure 23 is a graphical representation showing growth of *H. armigera* larvae on artificial diet containing NaPI and PotI. Growth of *H. armiger*a larvae fed on a cotton leaf artificial diet in the presence or absence of 0.26% (w/v) NaPI or 0.26% (w/v) NaPI plus 0.26% (w/v) PotI. The PotI was purified from potato tubers (var Russet Burbank), see Figure 22. Twenty five larvae were used on each diet. The weight of the larvae was measured at days 7, 10, 12, 14 and 17 post egg hatch. At day 17, larvae fed NaPI alone were 84% of the control and larvae fed NaPI and PotI were 34% of the control. Two larvae fed on the control diet died, seven larvae fed the NaPI diet died and six larvae fed on the NaPI plus PotI diet died.

Figure 24 is a representation showing the alignment of predicted amino acid sequence of StPotIA and StPotIB with members of the potato Inhibitor I family. ClustalW alignment of several members of the Potato Inhibitor I family. X67950: potato cDNA, (Beuning and Christeller, *Plant Physiol 102:* 1061, 1993), P01052: potato protein (Richardson and Cossins, *FEBS Letters, 52:* 161, 1975), M17108: potato genomic sequence (Cleveland *et al., Plant Mol. Biol. 8:* 199-207, 1987), K03290: tomato (Graham *et al., J. Biol. Chem., 260:* 6555-6560, 1985) Z12619: tobacco (Lindhorst *et al., Plant Mol. Biol. 21:* 985-992, 1993), X78988: maize (Jose Cordero *et al., Plant J. 6:* 141-150, 1994), EILXCH: leech (See Muller *et al., Hoppe-Seyler's Z. Physiol. Chem. 358:* 1105-1117, 1977). * P1 reactive

site. Both StPotIA and StPotIB are similar to other family members from potato. However, StPotIA has an additional four amino acids at position 41 to 44 that are also found in a wound induced PotI from tomato (K03290). StPotIB has a methionine at the P1 site which is common for potato isolates. StPotIA has an alanine at the P1 site which has not been reported for PotI isolates from potato, but is present in a PotI isolate from maize (X78988).

Figure 25 is a graphical representation showing purification of *E. coli* expressed StPotIA and StPotIB. StPot1A and StPotIB were purified using the N-terminal HIS tag fused to StPot1A and StPotIB and a metal affinity resin followed by RP-HPLC. Profile after separation by RP-HPLC. Protein was eluted with a linear gradient of 0-100% Buffer B (80% (v/v) acetonitrile, 0.1% (v/v) TFA) at a flow-rate of 1 ml/min over 60 min. **(A)** StPotIA, **(B)** StPotIB. StPotIA eluted in one major peak at 36 min retention time (60% Buffer B) and StPotIB eluted in one major peak at 25 min retention time (42% Buffer B). The peaks were analyzed by SDS-PAGE and stained with silver (insert in A). Lane 1 is StPotIA (pk1), lane 2 is StPotIB (pk2) and lane 3 is purified PotI from potato tubers. StPotIA and StPotIB have a different apparent mobility (10 kDa) to a mix of Pot1 isoforms isolated from tubers (6 kDa), due to the additional HIS-tag epitope at the N terminus.

Figure 26 is a graphical representation showing inhibition of NaPI-insensitive chymotrypsin by bacterially expressed StPotIA and StPotIB. Inhibition of the NaPI-insensitive chymotrypsin from the gut of *H. punctigera* with purified PotI. **(A)** substrate SA_2PFpNA, development time 30 min, **(B)** substrate SA_2PLpNA, 30 min incubation. Mix of PotI isoforms from potato tuber (circle), StPotIA (square), StPotIB (triangle). StPotIA, StPotIB and the PotI from potato tubers were good inhibitors of the NaPI- insensitive chymotrypsin.

Figure 27 is a graphical representation showing growth of *H. armigera* larvae on transgenic cotton expressing NaPI and PotI. Transgenic cotton cv Coker 315 was used in bioassays with *H. armigera*. Thirty larvae were fed leaves of either the control untransformed Coker 315, transgenic line 1 (NaPI), transgenic line 2 (StPot1A) or transgenic plant 3 (NaPI X StPot1A). The weight of the larvae was measured at day 7 post-

egg hatch. **(A)** Growth of larvae. At day 7, larvae fed leaves expressing NaPI were 86% of the weight of the control larvae fed untransformed leaves. Larvae fed leaves expressing StPotIA were 92% of the control and larvae fed leaves expressing both NaPI and StPotIA were 46% of the control. **(B)** The effect of ingestion of NaPI and PotI on gut trypsin and chymotrypsin activity. Gut from the larvae in each experiment were pooled and extracts prepared. All assays were performed in duplicate. Trypsin activity (black) was determined using BApNA substrate and chymotrypsin activity (grey) with SA_2PFpNA substrate. Units of activity are expressed as change in absorbance at 405 nm/min/ug gut extract protein. Trypsin activity was reduced, relative to the control, in the extracts from larvae fed leaves expressing NaPI and NaPI+StPotIA. Chymotrypsin activity was elevated in extracts from larvae fed leaves expressing NaPI or NaPI + StPotIA and reduced in extracts from larvae fed StPotIA alone.

Figure 28 diagrammatic representation showing the nucleotide sequence and deduced amino acid sequence from the HpF5 cDNA encoding the NaPI-insensitive chymotrypsin and the location of the oligonucleotide primers used to add an endoplasmic reticulum sequence to HpCh5. The FwBacRECH1 primer was used to add the first half of the ER signal sequence as well as a silent mutation, changing A to G to destroy the *Bam*HI cut site. The FwBacRECH2 primer added the remainder of the coding sequence for the ER signal as well as a *Bam*HI cut site to the 3' end of the sequence. The ER signal was added before the hexahistidine tag to enable purification of the expressed protein by metal affinity chromatography after cellular processing. The added amino acids are shaded in grey.

Figure 29 is a photographic representation showing expression of the chymotrypsin clone HpF5 in baculovirus infected insect cells. Expressed proteins were separated on 12.5% (w/v) SDS-PAGE gels and subjected to immunoblots with the α-HpCh5-antibody **(A)** RCDNA. Production of HpCh5 by HIGH FIVE (trademark) insect cells transfected with 20 μl of bacmid DNA. Controls; (pFastBacVector) insect cells transfected with bacmid DNA transposed with pFastBac vector without the HpF5insert; (blue colony) insect cells transfected with untransposed bacmid DNA; (cells alone) untransfected insect cells;

(Cellfectin) insect cells treated with CELFECTIN (registered trademark) alone; The positive control, + is bacterially expressed HpCh5 with a hexahistidine tag. (B) Production of HpCh5 by HIGH FIVE (trademark) insect cells infected with virus. RC 24, 48 and 72. Medium collected 24, 48 and 72 hours after infection with HpF5 recombinant virus. Controls 24, 48 and 72. Medium from cells treated with virus prepared from nontransfected bachmid (blue colony) for 24, 48 and 72 hours. Positive control is bacterially expressed HpCh5 with a hexahistidine tag.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated in part on the identification and cloning of a novel insect chymotrypsin molecule termed "HpCh5". cDNA encoding HpCh5 is referred to herein as "HpF5". The isolation of this molecule permits the identification and design of a range of products which are useful in controlling the growth, development and/or overall biological fitness of *Helicoverpa* spp. and other insects. These products generally act as antagonists of HpCh5 function or HpF5 gene expression and are useful as insect control agents.

The amino acid sequence of HpCh5 is set forth in SEQ ID NO:2. The nucleotide sequence of HpF5 is set forth in SEQ ID NOs:4 and 6.

Reference herein to "HpCh5" should be understood as a reference to all forms of HpCh5 including, for example, any peptide isoforms which arise from alternative splicing of HpF5 mRNA, mutants or polymorphic variants of HpCh5, any post-translation modified forms of HpCh5 or any non-post-translational modified forms of HpCh5 as well as any homolog in other insect species or strains. The term "HpCh5" also encompasses members in a HpCh5 family of chymotrypsin molecules. To the extent that it is not specified, reference herein to HpCh5 includes derivatives, homologs, analogs, chemical equivalents and mimetics thereof. Reference to HpCh5 also refers to any variant having at least 75% amino acid identity to SEQ ID NO:2 after optimal alignment. Examples of variants of HpCh5 include PI-sensitive variants such as those *inter alia* having an Arg 192 Gln or Arg 192 Asn substitution. Other variants include the N-terminal signal sequence of HpCh5 as defined in SEQ ID NO:3 and which is encoded by the nucleotide sequence set forth in SEQ ID NO:5. Such variants include a signal sequence comprising an amino acid sequence having at least about 75% similarity to SEQ ID NO:3 after optimal alignment or encoded by a nucleotide sequence having at least about 75% identity to SEQ ID NO:5 or a nucleotide sequence capable of hybridizing to SEQ ID NO:5 after optimal alignment.

Reference to "HpF5" should be understood as reference to all forms of HpF5 including any

cDNA isoform, genomic forms, mutants and polymorphic variants of HpF5 as well as any homologs from other species or strains of insect. The term "HpF5" also encompasses members of a HpF5 family of genes which encode HpCh5 or HpCh5-type chymotrypsins. To the extent that it is not specified, reference herein to HpF5 includes derivatives of HpF5 as well as a nucleotide sequence having at least about 75% identity to SEQ ID NO:4 or SEQ ID NO:6 or a nucleotide sequence capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions. The signal sequence of HpF5 as defined in SEQ ID NO:3 and encoded by SEQ ID NO:5 also encompasses variants thereof. As indicated above, such variants include a signal sequence having at least about 75% similarity to SEQ ID NO:3 after optimal alignment and/or being encoded by a nucleotide sequence capable of hybridizing to SEQ ID NO:5 under low stringency conditions and/or a nucleotide sequence having at least about 75% identity to SEQ ID NO:5 after optimal alignment.

Before describing the present invention detail, it is to be understood that unless otherwise indicated, the subject invention is not limited to specific formulations of components, manufacturing methods, administration regimens, or the like, as such may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to an "agent" or "antagonist" includes a single agent or antagonist as well as two or more agents or antagonists and so forth.

In describing and claiming the present invention, the following terminology is used in accordance with the definitions set forth below.

The terms "compound", "agent", "active agent" and "active" are used interchangeably herein to refer to a chemical compound which inhibits the activity of HpCh5 or the expression of a genomic gene corresponding to HpF5. The terms also encompass

agriculturally or horticultural active ingredients of those active agents specifically mentioned herein including but not limited to salts, esters, amides, prodrugs, active metabolites, analogs and the like. When the term "compound", "agent", "active agent" or "active" is used, then it is to be understood that this includes the agent *per se* as well as agriculturally or horticulturally acceptable, physiologically active salts, esters, amides, prodrugs, metabolites, analogs, etc. The term "compound" is not to be construed as a chemical compound only but extends to peptides, polypeptides and proteins as well as genetic molecules such as RNA, DNA and chemical analogs thereof.

The present invention contemplates, therefore, compounds useful in down-regulating the activity of HpCh5 or down-regulating expression of a genomic gene corresponding to HpCh5. The term "down-regulating" encompasses inhibition of HpCh5 activity. The inhibition of HpCh5 or reduction in its levels has the effect of reducing or retarding the growth of the insect. The inhibition of HpCh5 activity or HpF5 gene expression may occur by producing an inhibitor in a plant which is then consumed by the insect or the inhibitor may be topically applied to a plant or sprayed or otherwise dispersed to insects or a source of insects. In this regard, the plant may produce a nucleic acid molecule that interferes with HpF5 expression when consumed by the insect. Alternatively, the plant may produce a PI capable of inhibiting HpCh5. Still in a further alternative, the HpCh5 inhibitor is a non-proteinaceous chemical applied to the surface of a plant or taken up by the root system of a plant. Reference herein to a "plant" is not to exclude trees or cultured tissues (e.g. callus) from a plant (or tree).

Reference to compounds, agents and actives also includes combinations of compounds, agents or actives. Such combinations may be formulated in multi-part agricultural or horticultural compositions which are admixed together prior to dispersement or given sequentially.

The terms "effective amount", "insecticidal effective amount" and "insect-static effective amount" of an agent as used herein mean a sufficient amount of the agent to reduce or retard insect growth and development and/or to kill or inhibit the insect.

By "agriculturally acceptable" or "horticulturally acceptable" carrier, excipient or diluent is meant a vehicle comprised of a material that is not environmentally or otherwise undesirable to a plant or non-target insect. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like.

The terms "treating" and "treatment" as used herein in relation to plants or eradication of insects refer to reduction in severity of symptoms of insect infestation of a plant or the application of the agents to a group of insects resulting in retardation of their growth, development or biological fitness or wellbeing.

The compounds of the present invention may be large or small molecules, nucleic acid molecules (including antisense or sense molecules), peptides, polypeptides or proteins or hybrid molecules such as RNAi- or siRNA-complexes, ribozymes or DNAzymes.

The term "nucleic acid molecule" is also encompassed by the expression "genetic molecule" and includes hairpin constructs such as those which include RNAi-mediated post-transcriptional gene silencing or methylation-mediated silencing.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule or derivative, homolog or analog thereof comprising a nucleotide sequence encoding a novel chymotrypsin protein or a derivative, homolog or mimetic thereof wherein said chymotrypsin is insensitive to a PI of *N. alata*.

More particularly, the present invention is directed to a nucleic acid molecule or derivative, homolog or analog thereof comprising a nucleotide sequence encoding, or a nucleotide sequence complementary to a nucleotide sequence encoding, an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least about 75% or greater identity to SEQ ID NO:2 after optimal alignment or a nucleotide sequence set forth in SEQ ID NO:4 or SEQ ID NO:6 or a nucleotide sequence

having at least about 75% similarity or greater to SEQ ID NO:4 or SEQ ID NO:6 or a nucleotide sequence capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions.

Another aspect of the present invention provides an isolated chymotrypsin *Helicoverpa* ssp. wherein said chymotrypsin exhibits resistance to a PI from *N. alata* or a variant, derivative, homolog or analog of said chymotrypsin.

More particularly, the isolated chymotrypsin comprises an amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least about 75% similarity to SEQ ID NO:2 after optimal alignment.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and amino acid sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more)

polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res. 25:* 3389-3402, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the

reference manual accompanying the software. Similar comments apply in relation to sequence similarity. The term "similarity" is particularly useful to describe amino acid sequence comparisons. The term "identity" is particularly useful to describe nucleotide sequence comparisons.

Reference to at least about 75% identity or 75% similarity includes percentage identities and similarities greater than 75% such as 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%,. 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% and 100%.

Reference herein to a low stringency means from at least about 0 to at least about 15% (v/v) formamide (including 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% 11%, 12%, 13% and 14% (v/v) formamide) and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is at from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% (v/v) to at least about 30% (v/v) formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% (v/v) to at least about 50% (v/v) formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J. Mol. Biol. 5:* 109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem. 46:* 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 0.1% (w/v) SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 0.1% (w/v) SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% (w/v) SDS at a temperature of at least 65°C.

A further aspect of the present invention contemplates a nucleic acid molecule or derivative, homolog or analog thereof comprising a nucleotide sequence substantially as set forth in SEQ ID NO:4 or SEQ ID NO:6 or a nucleotide sequence having at least 75% or greater similarity to SEQ ID NO:4 or SEQ ID NO:6 or a derivative, homolog or analog thereof, or capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least 75% or greater identity to SEQ ID NO:2 after optimal alignment which nucleic acid molecule encodes a chymotrypsin which is insensitive to a proteinaceous inhibitor of *N. alata*.

Yet another aspect of the present invention contemplates nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:4 or SEQ ID NO:6. The nucleic acid molecule encoding HpCh5 is preferably a sequence of deoxyribonucleic acids such as a cDNA sequence or a genomic sequence. A genomic sequence may also comprise exons or introns. A genomic sequence may also include a promoter region or other regulatory regions. The present invention further contemplates isolated introns and exons of HpF5 such as those involved in genetic networking within a plant cell.

The nucleic acid molecule according to this aspect of the invention corresponds herein to HpF5. This cDNA has been determined, in accordance with the present invention, to encode a protein that defines a new family of chymotrypsins, family 5, within the group of chymotrypsin gene families, and this protein is referred to herein as HpCh5. Reference to "HpF5" also includes a genomic form of the gene. Within the *Helicoverpa punctigera* chymotrypsin gene families, there are varying levels of homology as shown in Table 2. Family 5 is exemplified by HpF5, and is most similar to family 2A at 73% and least similar to family 4 at <20%. Without intending to limit the instant invention in any way, HpCh5 is exemplified by two unique stretches of sequence in the N-terminal (F1 and F2) and by six amino acid substitutions relative to NaPI sensitive chymotrypsins. Five of these substitutions did not appear to fall into functionally significant regions, whereas the sixth

substitution is associated with one of the β-strands that forms a wall of the primary substrate-binding pocket. The location of this substitution and conversion to an arginine, from glutamine, is highly unusual for the S1 domain that is predominantly lined with non-polar residues that define chymotrypsin specificity

TABLE 2

Percentage protein sequence identity between members of the H. punctigera chmotrypsin gene family

***H. punctigera* chymotrypsin families**	***HpCh1AI***	***HpCh1BI***	***HpCh2A***	***HpCh2B***	***HpCh3A***	***HpCh3B***	***HpCh4AI***	***HpCh5***
HpCh1AI		90	54	53	58	59	<20	57
HpCh1BI			51	51	56	56	<20	55
HpCh2A				94	83	87	<20	73
HpCh2B					82	82	<20	72
HpCh3A						92	<20	70
HpCh3B							<20	72
HpCh4AI								<20

The present invention provides, therefore, an isolated protein having chymotrypsin activity which is not substantially inhibited by a PI from *N. alata*. Accordingly, another aspect of the present invention is directed to an isolated protein selected from the list consisting of:

(i) a novel chymotrypsin protein or a derivative, homolog or mimetic thereof wherein said chymotrypsin is insensitive to the proteinase inhibitors of *N. alata*;

(ii) a protein having an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least 75% or greater

identity to SEQ ID NO:2 after optimal alignment;

(iii) a protein encoded by a sequence of nucleotides substantially as set forth in SEQ ID NO:4 or SEQ ID NO:6 or a derivative, homolog or analog thereof or a nucleotide sequence having at least 75% similarity to SEQ ID NO:4 or SEQ ID NO:6 or a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least 75% or greater similarity to SEQ ID NO:2 after optimal alignment;

(iv) a protein encoded by a nucleic acid molecule or derivative, homolog or analog thereof comprising a nucleotide sequence substantially as set forth in SEQ ID NO:4 or SEQ ID NO:6 or a derivative, homolog or analog thereof, or capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 under low stringency conditions and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or a derivative, homolog or mimetic thereof or having at least 75% or greater similarity to SEQ ID NO:2 after optimal alignment.

(v) a novel chymotrypsin protein or a derivative, homolog or mimetic thereof that has an arginine substituted for an asparagine or glutamine in the primary substrate-binding pocket.

The present invention discloses the amino acid, and corresponding cDNA sequence of a novel chymotrypsin that is insensitive to the Type II serine proteinase inhibitors produced by solanaceous species such as *N. alata*. Therefore, this may be used as a target for agents to control insects carrying this insensitive proteinase. A number of compounds have been shown to inhibit the activity of HpCh5, and a list of these compounds as preferred embodiments is found in Table 3.

TABLE 3

Effect of various proteinase inhibitors on the activity of the NaPI- insensitive chymotrypsins from H. punctigera and bovine chymotrypsin

Inhibitor	Maximum concentration tested		% Inhibition		IC50	
	Insensitive μM	BC μM	Insensitive	BC	Insensitive μM	BC μM
NaPI	10	4	0%	100%	> 10	0.04
Chymostatin	0.05	0.1	100%	100%	0.004	0.004
Pot I	5	5	100%	100%	0.12	0.02
Bowman Birk	10	5	100%	100%	0.24	0.06
Lima bean	>20	5	89%	100%	3	0.2
SBTI	>20	>20	82%	94%	33	13
PMSF	1000	2000	100%	100%	33	13
Leupeptin	>4000	>4000	96%	52%	140	2400

SBTI, soybean trypsin inhibitor; PMSF, phenylmethyl sulphonyl fluoride; Bowman Birk, soybean Bowman Birk inhibitor; lima bean, lima bean trypsin inhibitor (Sigma); Pot I, potato proteinase inhibitor Type I. Bovine chymotrypsin (BC).

Without limiting the mode of action of any of these compounds to any one activity, 3-D modeling is used to investigate the binding ability of PotI and the peptide encoded by the C1 domain of NaPI to both NaPI-insensitive and NaPI-sensitive insect chymotrypsins.

The deduced amino acid sequences from the cDNA clones HpF2A (NaPI-sensitive) and HpF5 (NaPI-insensitive) were modeled on the structures of the *Solenopsis invicta* (fire ant) and *Bos taurus* (cow) chymotrypsins obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank.

The *Helicoverpa* chymotrypsins are predicted to adopt similar structures to those reported for all the chymotrypsin structures available in the data bank. The modeled structures have the classic serine protease fold consisting of two, six-stranded anti-parallel beta barrels with the catalytic triad located between the two domains. Certain surface loops are cleaved

in the mammalian chymotrypsins (loop 142), but remain intact within insect chymotrypsins. Therefore, the fire ant chymotrypsin structure (Botos *et al.*, *J. Mol. Biol. 298:* 895-901, 2000) was required to help refine the orientation of these surface loops in the *Helicoverpa* chymotrypsin models. Two surface loops, 60 and 142 are considerably larger in the *H. punctigera* chymotrypsins (Figures 15, 16).

C1 was modeled in complex with NaPI-sensitive and NaPI-insensitive chymotrypsins to investigate what residues in the NaPI-insensitive chymotrypsin might be involved in the loss of inhibitor binding. The structure of the chymotrypsin inhibitor C1 was previously determined by ^{1}H NMR (Nielson *et al.*, 1994, *supra*) but has not been determined in a proteinase complex. Therefore the related proteinase inhibitor PCI-1 from *Solanum tuberosum* in complex with Proteinase B from *Streptomyces griseus* (Greenblatt *et al.*, *J. Mol. Biol. 205:* 201, 1989) provided an appropriate basis guiding the alignment of the complexes. Energy minimization of the C1-chymotrypsin complexes revealed Arg192 (chymotrypsin numbering system) as the only likely candidate to cause such resistance from a possible 24 putative contact residues. The NaPI-insensitive chymotrypsin containing the Arg192 (Greer nomenclature, Greer, *Proteins* 7:317-34, 1990. Figure 15) residue in complex with C1 could not be properly energy minimized due to steric contacts between Arg192 and C1 whereas the Gln192 residue in the Na-PI sensitive chymotrypsin in complex with C1 caused no such problems due to its much smaller size. Figure 17 shows a close up view of the binding region surrounding Gln192 in the C1-HpF2A chymotrypsin complex. It is clear that Gln192 is not in conflict with any regions on the inhibitor molecule. However, comparison to the cognate Arg residue in the C1-HpCh5 chymotrypsin model demonstrates there is not enough space to accommodate this much larger residue (Figure 18) making contact with Thr5 and Ala9 in C1. Furthermore, modeling the StPot1A inhibitor into HpCh5 revealed that the NaPI-insensitive chymotrypsin could accommodate the Arg192 residue consistent with the inhibition of this chymotrypsin by StPot1A (Figure 19).

In summary, the NaPI-insensitive chymotrypsin from Helicoverpa species has an arginine in place of an asparagine or glutamine at position 192 that extends into the S1 binding

pocket and appears to interfere with C1 binding. Furthermore, it is clear that this Arg residue does not interfere with PotI binding, consistent with the observation that PotI is a much more efficient inhibitor of insect chymotrypsins than the NaPI inhibitors. Large quantities of the PotI inhibitor were purified from potato tubers (Figure 22) to evaluate the combined effect of NaPI and PotI on the growth of *H. armigera* larvae (Figure 23). Bioassays confirmed that PotI significantly enhances the activity of the NaPI inhibitors. Caterpillars fed NaPI and PotI in combination (0.26 and 0.34% (w/v), respectively) were 34% the size of control larvae at the fifth instar stage of development whereas caterpillars feeding on NaPIs alone were about 84% the size of the controls.

Therefore, another aspect of the present invention provides a method for modulating activity of the HpCh5 or a homolog or variant thereof in an insect, said method comprising contacting the HpCh5 protein or its homolog or variant with an effective amount of an agent for a time and under conditions sufficient to decrease or increase HpCh5 activity.

Yet another aspect of the present invention provides a method for modulating expression of HpF5 or homolog or variant in an insect, said method comprising contacting HpF5 or its homolog or variant with an effective amount of an agent for a time and under conditions sufficient to decrease or increase HpF5 expression.

The preferred insects targeted in accordance with these and other aspects are species of *Helicoverpa* and other Lepidopteran species. In addition, plants to be protected include those sensitive to *H. armigera, H. punctigera, H. zea and H. virescens*. Such plants include the *H. armigera* sensitive plants such as cotton, sweet corn, tomato, tobacco, piniento, potato, sunflower, citrus, plums, sorghum, leeks, soybean, alfalfa, beans, pidgeon peas, chick peas, artichokes, curcurbits, lettuce, *Dianthus* (an ornamental plant), geraniums, cape gooseberry, maize, flax and linseed, alfalfa, lupins, broad beans, garden peas, peanuts, canola, snapdragons, cherry, sunflower, pot marigolds and *Helichrysum* (an ornamental plant)

Other plants contemplated herein include cereals (such as wheat, barley, oats, triticale,

etc.), horticultural plants (e.g. apples, carrots, onions, etc.), ornamental plants (such as orchids, roses, petunias, etc.) and trees.

The present invention contemplates, therefore, methods of screening for compounds which inhibit or act as antagonists of HpCh5 activity or HpF5 gene expression. For example, one method involves contacting a candidate compound with HpCh5. The screening procedure includes assaying (i) for the presence of a complex between the compound and HpCh5, or (ii) an alteration in the expression levels of HpF5 cDNA or genomic DNA. One form of assay involves competitive binding assays. In such competitive binding assays, HpCh5 is typically labeled. Free HpCh5 is separated from any putative complex and the amount of free (i.e. uncomplexed) label is a measure of the binding of the agent being tested to bind to HpCh5. One may also measure the amount of bound, rather than free, HpCh5. It is also possible to label the compound rather than HpCh5 and to measure the amount of compound binding to target in the presence and in the absence of the compound being tested. Such compounds may inhibit HpCh5. A similar approach may be adopted for compounds which bind to and inhibit HpF5 or mRNA transcripts thereof.

Another technique for agent screening provides high throughput screening for compounds having suitable binding affinity to HpCh5 and is described in detail in Geysen (International Patent Publication No. WO 84/03564). Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with HpCh5 and washed. Bound HpCh5 molecules are then detected by methods well known in the art. This method may be adapted for screening for non-peptide, chemical entities. This aspect, therefore, extends to combinatorial approaches including phage display to screen for HpCh5 antagonists.

Purified HpCh5 can be coated directly onto plates for use in the aforementioned agent screening techniques. However, non-neutralizing antibodies to HpCh5 may also be used to immobilize HpCh5 on the solid phase.

Live animals such as *H. armigera* and/or *H. punctigera* may also be used in feeding trials to find potential inhibitors.

The present invention also contemplates the use of competitive agent screening assays in which neutralizing antibodies capable of specifically binding HpCh5 compete with a test compound for binding to HpCh5 or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants of HpCh5.

Yet another useful source of analogs of compounds which are chemically modified may be used to induce feed-back inhibition of biochemical or genetic pathways for generating authentic HpCh5.

Analogs of HpCh5 contemplated herein include but are not limited to modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on HpCh5.

Examples of side chain modifications of HpCh5 contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitization, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphonyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 4.

TABLE 4

Codes for non-conventional amino acids

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgln
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug

D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr

D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe

N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogs by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

Such analogs, especially if they retain activity or even the HpCL5 molecule itself may have indistinct applications such as in washing powder or as in a stain removal formulation.

Another aspect of the present invention contemplates any compound which binds or otherwise interacts with HpCh5 or its derivatives or variants or which induces feed-back inhibition of HpCh5 synthesis resulting in down-regulation of HpCh5 activity or levels.

The present invention is also useful for screening for other compounds which reduce expression of HpF5. A variety of agent screening techniques may be employed such as those described herein and in International Publication No. WO 97/02048.

A compound antagonist includes a variant of HpCh5 such as a variant comprising an analog amino acid residue as indicated above. In one embodiment, the target is the HpCh5

polypeptide. The term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product, thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. This term also does not exclude modifications of the polypeptide, for example, glycosylations, aceylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids such as those given in Table 4) or polypeptides with substituted linkages.

A substance identified as an antagonist of HpCh5 function or HpF5 gene activity may be a peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many agricultural or horticultural purposes due to their perceived stability.

There are several steps commonly taken in the design of a mimetic-type antagonist of HpCh5 from a compound. First, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptides are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "agrichemicaphore".

Once the agrichemicaphore has been found, its structure is modeled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, x-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a agrichemicaphore, rather than the bonding between atoms) and other techniques can be used in this modeling process.

In a variant of this approach, the three-dimensional structure of HpCh5 and a compound binding it. This can be especially useful where HpCh5 or its antagonist change conformation on binding, allowing the model to take account of this in the design of the

mimetic. Modeling can be used to generate inhibitors which interact with the linear sequence or a three-dimensional configuration.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto it can conveniently be selected so that the agrichemicaphore is easy to synthesize and is likely to be agriculturally or horticulturally acceptable. Alternatively, where the agrichemicaphore is peptide-based, further stability can be achieved by cyclizing the peptide, increasing its rigidity. The agrichemicaphore or agrichemicaphores found by this approach can then be screened to see whether they have HpCh5 antagonistic property, or to what extent they exhibit it. Further optimization or modification can then be carried out to arrive at one or more final agents for testing.

Yet another aspect of the present invention provides a method for detecting an agent capable of binding or otherwise associating with a HpCh5 binding site or functional equivalent thereof said method involving the use of *in-silico* 3-D modeling to identify compounds that bind to HpCh5 and specifically, are not interfered with by Arginine 192.

The goal of rational HpCh5 antagonist design is to produce structural analogs of HpCh5 or of small molecules with which HpCh5 interacts (e.g. an antagonist or inhibitor) in order to fashion agents which are, for example, more inhibitory of HpCh5. See, e.g. Hodgson (*Bio/Technology 9:* 19-21, 1991). In one approach, one first determines the three-dimensional structure of HpCh5 by x-ray crystallography, by computer modeling or, most typically, by a combination of approaches. Useful information regarding the structure of a polypeptide may also be gained by modeling based on the structure of chymotrypsins. An example of rational drug design is the development of HIV protease inhibitors (Erickson *et al.*, *Science 249:* 527-533, 1990). In addition, target molecules may be analyzed by an alanine scan (Wells, *Methods Enzymol. 202:* 2699-2705, 1991). In this technique, an amino acid residue is replaced by Ala and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

In addition, compounds including antagonists may be directed to particular locations or regions or domains or HpCh5.

It is also possible to isolate a HpCh5-specific antibody and then to solve its crystal structure. In principle, this approach yields an agricore upon which subsequent agent design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional antibody. As a mirror image of a mirror image, the binding site of the anti-ids is expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced banks of peptides. Selected peptides would then act as the agrichemicaphore.

Chymotrypsin clone HpF2B is expressed in *E. coli* fused to a six histidine (6.H) tag at the C-terminus and is purified to homogeneity on Talon metal affinity resin (Figure 20) for injection into a rabbit for production of polyclonal antibodies. N-terminal sequencing of the purified product confirmed the expression of the chymotrypsin HpCh2B. After the fourth boost with antigen, the serum is collected and tested on protein blots of bacterially expressed protein and unfractionated gut extracts. The antibody detected the full-length recombinant chymotrypsin at a dilution of 1 in 2500 as well as several break-down products. Unfractionated gut extract and a sample of protein bound to the C1 affinity column were also stained with the anti-HpCh2B antibody which detected the mature native form of the enzyme.

Purified 6H.HpCh2B is used to test the detection limit of anti-HpCh2B antibody by comparison of immunoblots to silver stained SDS-PAGE gels. The antibody detected 20 ng of bacterially expressed chymotrypsinogen and also recognized the mature form of the native chymotrypsin isolated from gut of *H. punctigera*.

The cDNA (HpF5) encoding the NaPI-insensitive chymotrypsin (HpCh5) is expressed in *E. coli* in a similar manner except the six-histidine tag is fused to the N-terminus of the

expressed protein. The polyclonal antiserum that is raised against the bacterially expressed chymotrypsin HpCh2B did not cross react with bacterially expressed NaPI- insensitive chymotrypsin (HpCh5) on protein blots (Figure 21). Likewise the antiserum raised against HpCh5 did not bind to HpCh2B. This indicates that these antisera can be used to specifically distinguish between and monitor levels of the NaPI -insensitive and sensitive chymotrypsins in unfractionated gut extracts

Accordingly, still another aspect of the present invention is directed to antibodies to HpCh5 and HpCh2B including catalytic antibodies.

In another aspect of the present invention, a method is provided for the isolation of and separation of individual isoforms of chymotrypsin, said method consisting of:

(i) affinity chromatography of insect gut extracts initially with benzamidine-sepharose to bind trypsins;

(ii) further affinity chromatography of the unbound proteins using immobilized *N. alata* serine proteinase inhibitor C1 to bind all NaPI inhibitable chymotypsins; and

(iii) affinity chromatography of the eluate from (ii) with immobilized PotI and PotII or chymostatin to bind the remainder. The putative NaPI-insensitive chymotrypsins are then eluted with 8 M urea.

The present invention extends to a genetic approach to down-regulating expression of an HpF5 or its homologs or variants. Such an approach uses nucleic acid molecules or molecules having a genetic component (e.g. RNAi) to induce pre- or post-transcriptional gene silencing.

The terms "nucleic acids", "nucleotide" and "polynucleotide" include RNA, cDNA, genomic DNA, synthetic forms and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized

nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog (such as the morpholine ring), internucleotide modifications such as uncharged linkages (e.g. methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g. phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g. polypeptides), intercalators (e.g. acridine, psoralen, etc.), chelators, alkylators and modified linkages (e.g. α-anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence *via* hydrogen binding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

Antisense polynucleotide sequences, for example, are useful in silencing transcripts of HpF5. Furthermore, polynucleotide vectors containing all or a portion of HpF5 gene locus may be placed under the control of a promoter in either the sense or antisense orientation and introduced into a cell. Expression of such a sense or antisense construct within a cell interferes with target transcription and/or translation. Furthermore, co-suppression (i.e. using sense-suppresion) and mechanisms to induce RNAi or siRNA may also be employed. Alternatively, antisense or sense molecules may be directly administered. In this latter embodiment, the antisense or sense molecules may be formulated in a composition and then administered by any number of means to target cells.

A variation on antisense and sense molecules involves the use of morpholinos, which are oligonucleotides composed of morpholine nucleotide derivatives and phosphorodiamidate linkages (for example, Summerton and Weller, *Antisense and Nucleic Acid Drug Development 7:* 187-195, 1997). Such compounds are injected into embryos and the effect of interference with mRNA is observed.

In one embodiment, the present invention employs compounds such as oligonucleotides and similar species for use in modulating the function or effect of nucleic acid molecules

encoding HpCh5, i.e. the oligonucleotides induce transcriptional or post-transcriptional gene silencing. This is accomplished by providing oligonucleotides which specifically hybridize with one or more nucleic acid molecules encoding the inhibitor. The oligonucleotides may be provided directly to a cell or generated within the cell. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding HpCh5" have been used for convenience to encompass DNA encoding the inhibitor, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. The hybridization of a compound of the subject invention with its target nucleic acid is generally referred to as "antisense". Consequently, the preferred mechanism believed to be included in the practice of some preferred embodiments of the invention is referred to herein as "antisense inhibition." Such antisense inhibition is typically based upon hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard, it is presently preferred to target specific nucleic acid molecules and their functions for such antisense inhibition.

The functions of DNA to be interfered with can include replication and transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. The functions of RNA to be interfered with can include functions such as translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA.

In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine

are complementary nucleobases which pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

An antisense compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid sequences under conditions in which specific binding is desired.

"Complementary" as used herein, refers to the capacity for precise pairing between two nucleobases of an oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

According to the present invention, compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds which hybridize to at least a portion of the target nucleic acid. As such, these compounds may be introduced in the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid. One

non-limiting example of such an enzyme is RNAse H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNAse H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products.

In the context of the subject invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

While oligonucleotides are a preferred form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those herein described.

The open reading frame (ORF) or "coding region" which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is a region which may be effectively targeted. Within the context of the present invention, one

region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA *via* a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. It is also preferred to target the 5' cap region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns", which are excised from a transcript before it is translated. The remaining (and, therefore, translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e. intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred target sites. mRNA transcripts produced *via* the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It is also known that introns can be effectively targeted using antisense compounds targeted to, for example, DNA or pre-mRNA.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside.

For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may, therefore, fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

For topical delivery of antisense compounds, these oligonucleotides may contain modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the

nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

In an alternative embodiment, genetic constructs including DNA "vaccines" are used to generate antisense or sense molecules in plant cells. Furthermore, many of the preferred features described above are appropriate for sense nucleic acid molecules.

A further aspect of the present invention relates to a method for control of insect populations, said method comprising administering to insects an effective amount of an agent for a time and under conditions sufficient to inhibit the expression of HpF5 or sufficient to inhibit the activity of HpCh5, wherein said modulation results in reduction of the biological fitness of said insects.

In one preferred embodiment of the present invention, the agent is one that can bind to the primary substrate binding pocket of HpCh5, and not be interfered with by the arginine residue found at position 192.

Reference to a "reduction of biological fitness" should be understood to be changes in the insect including, but not limited to, changes in body mass and/or viability. Preferably, these changes are understood as reductions in both body mass and/or viability. A reduction in biological fitness, therefore, includes a reduction in their growth and development.

In yet another aspect the present invention provides a method for detecting an agent capable of modulating the function of HpCh5 or functional equivalent or derivative thereof, said method comprising administering to an insect containing said HpCh5 or functional equivalent or derivative thereof with a putative agent and detecting an altered activity phenotype associated with modulation of function of HpCh5 or its functional equivalent or derivative.

Reference to "administration" of the modulator to HpCh5 refers to delivery of the modulating agent in any convenient means. In the agricultural setting this is likely to

include, but not be limited to:-

(i) the delivery of the agent as the active ingredient in a spray or powder formulation; or

(ii) the production of the agent either as a protein or the product of a metabolic pathway in a plant.

In a preferred embodiment of the invention, the administration of the agent is *via* the introduction of a nucleic acid encoding said agent into a plant for subsequent expression and production of the agent. Another preferred embodiment is the formulation of a spray or powder with said agent as the active ingredient.

"Introduction" of the agent is to be understood to cover all means known to those in the art of making genetic changes in a plant. These include, but are not limited to plant transformation methods such as particle bombardment, *Agrobacterium*-mediated transformation, electroporation and viral transfection; plant-breeding techniques and mutagenesis of native plant genes.

Accordingly, in the context of the present invention, nucleic acid molecules encoding an antisense or sense form of HpF5 or encoding an inhibitor of HpCh5 activity or HpF5 expression is operably linked to a promoter, generally in a vector or other suitable medium for introduction to a plant genome. Alternatively, an existing PI may be cloned and modified to render it active against HpCh5.

The present invention further provides a genetically modified plant comprising cells which are capable of producing an antagonist of HpCh5 or HpF5 gene expression. In one particularly useful example, cotton or other crop plants are engineered to produce PotI or a combination of PotI and NaPI. Reference herein to "PotI" and "NaPI" includes reference to derivatives, variants and homologs including modifications to one or more domains in PotI or NaPI.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', or a structural gene region, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell.

Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the sense molecule and/or to alter the spatial expression and/or temporal expression of said sense molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense molecule, thereby conferring copper inducibility on the expression of said molecules.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations, it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e. the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a

heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e. the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

The promoter may regulate the expression of HpF5 or its variant or homolog constitutively, or differentially with respect to cell, the tissue or organ in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or pathogens, or metal ions, amongst others.

Preferably, the promoter is capable of regulating expression of a nucleic acid molecule in a plant cell, tissue or organ, at least during the period of time over which the target gene is expressed therein and more preferably also immediately preceding the commencement of detectable expression of the HpF5gene in said cell, tissue or organ.

Accordingly, strong constitutive promoters are particularly useful for the purposes of the present invention or promoters which may be induced by virus infection or the commencement of HpF5 gene expression.

Plant-operable promoters are particularly preferred for use in the construct of the present invention. Examples of suitable promoters include pCaMV 35S (Fang *et al., Plant Cell 1:* 141-150, 1989), PGEL1 (Hajdukiewicz *et al., Plant Mol. Biol. 25:* 989-994, 1994), class III chitinase (Samac and Shah, *Plant Cell 3:* 1063-1072, 1991), *pin2* (Keil *et al., EMBO J. 8:* 1323-1330, 1989), PEP carboxylase (Pathirana *et al., Plant J. 12:* 293-304, 1997; MAP kinase (Schoenbeck *et al., Molec. Plant-Microbe Interact*, 1999), MSV (Legavre *et al.*, In: Vth International Congress of Plant Molecular Biology, Singapore, 1997), p*ltp* (Hsu *et al., Plant Sci. 143:* 63-70, 1999), p*mpi* (Cordero *et al.*, In: General Meeting of the International Program on Rice Biotechnology of the Rockefeller Foundation, Malacca, Malaysia, 1997) or glutamin synthase (Pujade-Renaud *et al., Plant Physiol. Biochem. 35:* 85-93, 1997).

In the present context, the terms "in operable connection with" or "operably under the control" or similar shall be taken to indicate that expression of the nucleic acid molecule is under the control of the promoter sequence with which it is spatially connected; in a cell, tissue, organ or whole plant.

The construct preferably contains additional regulatory elements for efficient transcription, for example, a transcription termination sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences generally containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants or synthesized *de novo*.

As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used.

Examples of terminators particularly suitable for use in the synthetic genes of the present invention include the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the CYC1 terminator, ADH terminator, SPA terminator, nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens*, the terminator of the cauliflower mosaic virus (CaMV) 35S gene, the *zein* gene terminator from *Zea mays*, the Rubisco small subunit gene (SSU) gene terminator sequences, subclover stunt virus (SCSV) gene sequence terminators, any *rho*-independent *E. coli* terminator, or the *lacZ* alpha terminator, amongst others.

In a particularly preferred embodiment, the terminator is the SV40 polyadenylation signal or the HSV TK polyadenylation signal which are operable in animal cells, tissues and organs, octopine synthase (OCS) or nopaline synthase (NOS) terminator active in plant cells, tissue or organs, or the *lacZ* alpha terminator which is active in prokaryotic cells.

Those skilled in the art will be aware of additional terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

Means for introducing (i.e. transfecting or transforming) cells with the constructs are well-known to those skilled in the art.

The constructs described *supra* are capable of being modified further, for example, by the inclusion of marker nucleotide sequences encoding a detectable marker enzyme or a functional analogue or derivative thereof, to facilitate detection of the synthetic gene in a cell, tissue or organ in which it is expressed. According to this embodiment, the marker nucleotide sequences will be present in a translatable format and be expressed.

Those skilled in the art will be aware of how to produce the constructs described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular, it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or a plant cell or an animal cell.

The constructs of the present invention may be introduced to a suitable cell, tissue or organ without modification as linear DNA, optionally contained within a suitable carrier, such as a cell, virus particle or liposome, amongst others. To produce a genetic construct, a nucleic acid (e.g. HpF5) is inserted into a suitable vector or episome molecule, such as a bacteriophage vector, viral vector or a plasmid, cosmid or artificial chromosome vector which is capable of being maintained and/or replicated and/or expressed in the host cell, tissue or organ into which it is subsequently introduced.

Accordingly, a further aspect of the invention provides a genetic construct which at least comprises a genetic element as herein described and one or more origins of replication and/or selectable marker gene sequences.

Usually, an origin of replication or a selectable marker gene suitable for use in bacteria is physically-separated from those genetic sequences contained in the genetic construct which are intended to be expressed or transferred to a plant cell, or integrated into the genome of a plant cell.

As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell on which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin-resistance gene (Amp^r), tetracycline-resistance gene (Tc^r), bacterial kanamycin-resistance gene (Kan^r), the zeocin resistance gene (Zeocin is a drug of the bleomycin family which is trade mark of InVitrogen Corporation), the *AURI-C* gene which confers resistance to the antibiotic aureobasidin A, phosphinothricin-resistance gene, neomycin phosphotransferase gen (*npt*II), hygromycin-resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein-encoding gene or the luciferase gene, amongst others.

Preferably, the selectable marker gene is the *npt*II gene or Kan^r gene or green fluorescent protein (GFP)-encoding gene.

Those skilled in the art will be aware of other selectable marker genes useful in the performance of the present invention and the subject invention is not limited by the nature of the selectable marker gene.

The present invention extends to all genetic constructs essentially as described herein, which include further genetic sequences intended for the maintenance and/or replication of said genetic construct in prokaryotes or eukaryotes and/or the integration of said genetic construct or a part thereof into the genome of a eukaryotic cell or organism.

Standard methods described *supra* may be used to introduce the constructs into the cell, tissue or organ, for example, liposome-mediated transfection or transformation, transformation of cells with attenuated virus particles or bacterial cells, cell mating, transformation or transfection procedures known to those skilled in the art.

Additional means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, direct DNA uptake into protoplasts, PEG-mediated uptake to protoplasts, microparticle bombardment, electroporation, microinjection of DNA, microparticle bombardment of tissue explant or cells, vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue.

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 µm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

In a further embodiment of the present invention, the genetic constructs described herein are adapted for integration into the genome of a cell in which it is expressed. Those skilled in the art will be aware that, in order to achieve integration of a genetic sequence or genetic

construct into the genome of a host cell, certain additional genetic sequences may be required. In the case of plants, left and right border sequences from the T-DNA of the *Agrobacterium tumefaciens* Ti plasmid will generally be required.

The present invention further extends to an isolated cell, tissue or organ comprising the constructs or parts thereof. The present invention extends further to regenerated tissues, organs and whole organisms derived from said cells, tissues and organs and to propagules and progeny thereof as well as seeds and other reproductive material.

For example, plants may be regenerated from transformed plant cells or tissues or organs on hormone-containing media and the regenerated plants may take a variety of forms, such as chimeras of transformed cells and non-transformed cells; clonal transformants (e.g. all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissue (e.g. a transformed root stock grafted to an untransformed scion in citrus species). Transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques.

Plants contemplated herein include cotton, sweet corn, tomato, tobacco, piniento, potato, sunflower, citrus, plums, sorghum, leeks, soybean, alfalfa, beans, pidgeon peas, chick peas, artichokes, curcurbits, lettuce, *Dianthus* (an ornamental plant), geraniums, cape gooseberry, maize, flax and linseed, lupins, broad beans, garden peas, peanuts, canola, snapdragons, cherry, sunflower, pot marigolds, *Helichrysum* (an ornamental plant), wheat, barley, oats, triticale, carrots, onions, orchids, roses and petunias

Another aspect of the present invention relates to the insensitive chymotrypsin as a selectable marker for the transformation of insects. At present, methods for the germ-line transformation of insects involves injection of insect embryos with a genetic construct comprising a transposable element, the gene of interest and a selectable marker. Somatic

transformation of insects can also be achieved using viral vectors that include the gene of interest and a selectable marker (Peloquin *et al., J. Cot. Sci. 5:* 114-120, 2001). Presently, most insect transformation is done using white-eye mutants of the insect, to allow the detection of the commonly used selectable markers. Typically, selectable markers are genes that complement the white-eye mutation or Enhanced Green Fluorescent Protein (EGFP). In *Drosophila*, the white eye mutation is caused by mutant alleles such as the ϖ^{1118} allele. These individuals can be returned to normal (red) eye pigmentation via the introduction of an allele conferring normal eye pigmentation such as *white* (Lidholm *et al., Genetics 134:* 859-868, 1993) or *miniwhite* (Lozovskaya *et al., Genetics 142:* 173-177, 1996). The reversion of ϖ^{1118} mutants to normal eye pigmentation acts as the marker for introduction of the vector. In a similar way, EGFP has been used to indicate the presence of a vector. Again, insects with non-pigmented eyes are used, and EGFP expression is detected in the eyes of these insects (Hediger *et al., Insect Mol. Biol. 10:* 113-119, 2001).

The selectable markers commonly used in the art require dissection of the insects to examine the eyes for either pigmentation or EGFP fluorescence, which is time consuming and requires destructive sampling of the insects. The present invention provides a means for the selection of transformed individuals without the need for insect dissection or inspection of individual insects. This would allow the recovery of live transformants and provides a non-laborious means of screening large numbers of putative transformants at one time. In addition, the present invention provides a means for the selection of transformants that does not rely on the availability of white-eye mutants.

The present invention contemplates the use of HpF5 or a derivative, homolog or analog thereof encoding a NaPI-insensitive chymotrypsin, as a selectable marker in an insect transformation vector. This vector, comprising HpF5, would have utility for the selection of transformants for any insect that is susceptible to the C1 serine proteinase inhibitor of *N. alata*. The contemplated insects may be naturally resistant to C1, or may be NaPI-susceptible mutants or genetically modified NaPI susceptible strains of naturally NaPI-resistant insects. In a particularly preferred embodiment of the invention the insect host of the said vector would be Lepidopteran.

HpF5 or a derivative, homolog or analog thereof encoding an NaPI-insensitive chymotrypsin could be incorporated into any insect transformation vector using common molecular biology techniques known to those in the art. Upon transformation the insect would transcribe HpF5 and produce HpCh5, the NaPI-insensitive chymotrypsin. Transformed individuals could then be selected by incorporation of the C1 proteinase inhibitor of *N. alata* into the diet of the insects. In this case individuals that did not carry the insensitive chymotrypsin encoded by HpF5 in the vector would die, and those that did carry the vector encoding HpF5 would be insensitive to C1.

Accordingly the present invention provides insect transformation vectors including baculovirus vectors comprising HpF5 or a derivative, homolog or analog thereof, as a selectable marker. The vector may be used for any purpose in the insect. Non-limiting examples include: gene cloning, gene expression and gene knockouts. Specific examples of insect transformation vectors into which HpF5 could be incorporated as a selectable marker include, but are not limited to: those that utilize the *piggyBac* mobilizable element (Hediger *et al.*, 2001, *supra*); P-element based vectors (Cripps *et al.*, *J. Cell Biol. 126:* 689-699, 1994); *hobo* element based vectors (Lozovskaya *et al.*, 1996, *supra*); *mariner* element based vectors (Lidholm *et al.*, 1993, *supra*); and viral vectors such as pTE/3'2J (Peloquin *et al.*, 2001, *supra*).

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Effect of ingestion of the N. alata proteinase inhibitors on growth and development of Helicoverpa species

The effect of the *N. alata* PIs is examined on the digestive enzymes and the growth and development of *Helicoverpa punctigera* and *Helicoverpa armigera* larvae (Heath *et al., J. Insect Physiol. 43:* 833-842, 1997). The PIs suppressed total gut protease activity by 73% in an *in vitro* assay using ^{14}C-casein as substrate. When incorporated into an artificial diet the PIs retarded the growth and development of both *H. punctigera* (Heath *et al.*, 1997, *supra*) (Figure 2A and 2B) and *H. armigera*. Similar results are obtained when larvae from both species were fed on transgenic tobacco (*N. tabacum*) expressing the *N. alata* PIs at levels of 0.2% -0.5% soluble protein (Heath *et al.*, 1997, *supra*).

Ingestion of NaPIs (C1, C2 and T1-T4) changes the relative activity of the trypsins and chymotrypsins in the gut and faeces of *H. punctigera* larvae. Trypsin activity in the gut and the faeces is substantially lowered or abolished after exposure to NaPIs, whereas chymotrypsin activity is often unaffected or enhanced (Figures 2C and 2D). The lack of trypsin activity in the faeces of the NaPI fed larvae was not due to decreased production of trypsin. Indeed protein blots were used to demonstrate that these insects had overproduced trypsin in response to the PIs, but it had been totally inactivated (Figure 2E).

Interestingly, the chymotrypsin produced after ingestion of NaPIs was not inhibitable by the NaPIs, whereas some of the chymotrypsin produced by control insects (not exposed to NaPIs) was inhibitable (Figure 2F). In control insects the degree of inhibition varied between individuals, and ranged from about 75% to no inhibition at all. This suggested that *Helicoverpa* larvae produce different classes of chymotrypsins; some that are inhibitable by the NaPIs (PI-sensitive) and some that are not inhibitable (PI-insensitive). Thus, it appears ingestion of NaPIs inhibits the trypsin activity in the gut of *Helicoverpa* larvae, but larvae may not be severely impacted if they produce NaPI-insensitive chymotrypsins. In a subsequent experiment, the gut contents were removed from *H. punctigera* larvae that had been raised on a haricot bean diet without added proteinase inhibitors. The gut

chymotrypsin activity was inhibited about 80% by the NaPI inhibitors (C1, C2, T1-T4) and 70% by C1 alone (Figure 3). The Bowman Birk and trypsin inhibitors from Soybean and the lima bean trypsin inhibitor also failed to abolish all the chymotrysin activity, whereas activity was totally inhibited by the potato PotI inhibitor and chymostatin. Using this information a series of affinity columns were prepared for identification and purification of the NaPI-insensitive and -sensitive chymotrypsins from the gut of *H. punctigera* larvae.

Bioassays with Helicoverpa larvae on artificial diets

Haricot bean diet

Helicoverpa punctigera larvae were raised on artificial diets based on Haricot beans (Teakle *et al., Journal of Invertebrate Pathology 46:* 166-173, 1985). One litre of diet was composed of 58.5 g Haricot beans, 14 g agar, 700 ml water, 35 g Tortula yeast, 50 g wheatgerm, 3.5 g ascorbic acid, 1.1 g sorbic acid, 2.2 g p-hydroxybenzoic acid methyl ester, 0.2 g ampicillin, 0.2 g streptomycin, 16 mg prochloraz. The beans were soaked overnight in water, drained and homogenized to a fine paste. Wheatgerm, yeast and 300 ml of water were added. The agar was dissolved in 400 mL of boiling water and added to the mixture. The mixture was cooled to 50°C before the addition of the remaining ingredients. The blended diet was poured into trays and after setting was used immediately or stored at -20°C for no longer than two weeks. The test diet was supplemented with the NaPI (0.26% (w/v)) and the control diet had an equivalent amount of casein. Twenty newly emerged neonates were added to each diet and mortality was recorded every two days. Weight gain was recorded at the sixth day and then every second day thereafter. The larvae were reared in 1.5 ml eppendorf microfuge tubes (one larva/tube) until day eight when they were transferred to individual plastic containers with lids (SOLO [trademark] plastic portion cups, 28 mL). Larvae were fed small amounts of diet (40 mg) initially that was replaced as required to provide a continuous supply. The larvae were kept in a temperature controlled room at 25 ± 1°C, 16:8 (L:D).

Cotton leaf diet

Cotton leaf artificial diet was prepared from fresh young leaves from cotton plants (cultivar Coker 315) which were grown in an insect-free and insecticide-free temperature controlled

cabinet at 26°C (±2°C) with a light regime of 16:8 (L:D). Following picking, the leaves were immediately frozen in liquid nitrogen and freeze dried. After drying, the leaves were ground to a fine powder in a mortar and pestle. The cotton leaf artificial diet was prepared in the same manner as haricot bean artificial diet using a recipe modified from potato leaf artificial diet (Gatehouse *et al.*, *J. Insect Physiol. 45 (6)*, 545-558, 1999). One hundred grams of cotton leaf artificial diet contained 3 g of cotton leaf powder, 0.08 mL linseed oil, 2 g yeast, 0.016 mL wheatgerm oil, 2.4 g wheat germ, 0.028 g ampicillin, 3.2 g ascorbic acid, 0.028 g streptomycin, 0.08 g sorbic acid, 3.2 g agar, 0.16 g paraben (mould inhibitor) plus NaPI or casein to required % ((w/v)).

Preparation of individual gut and frass extracts

All work was performed at 4°C. Each gut was dissected from the larva and placed in a 1.5 mL microfuge tube containing 500 μL of ice-cold 10 mM Tris-HCl, pH 8. Gut and contents were homogenized using a micropestle (Eppendorf). Insoluble material was removed by centrifugation at 13,000 g for 4 min and the supernatant was stored at -80°C. Frass extracts were prepared in the same manner using 200 mg frass/mL buffer. Total protein concentration was determined using the Bradford method (Bradford, *Anal Biochem 72:* 248-254, 1976) with reagents from Bio-Rad and BSA as a standard.

Trypsin and chymotrypsin activity

Gut proteinase activity was determined at pH 10 in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS buffer) using the chromogenic substrate N-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) for trypsin and N-succinyl-L-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SA_2PFpNA) for chymotrypsin activity. Substrates were freshly prepared as 1 mM solutions in 10% ((w/v)) N,N-dimethylformamide (DMF), 50 mM CAPS buffer, pH 10. The assays were performed in duplicate or triplicate following the method of Heath *et al, Eur.J.Biochem.230 (1):* 250-257. Blanks, without enzyme, were used to account for any spontaneous breakdown of substrates. The release of *p*-nitroanilide was recorded at 405 nm after 30 min at 30°C on a SpectraMax 250 microtitre plate reader (Molecular Devices).

Inhibition of trypsin and chymotrypsin activity by NaPI

Trypsin and chymotrypsin inhibition assays were conducted using the standard trypsin and chymotrypsin assays described above except samples were pre-incubated with T1 or C1 inhibitor (80nM) for 30 min at 30°C prior to the addition of substrate to initiate the reaction. NaPI monomers T1 and C1 were HPLC purified as described by Heath *et al.*, 1999, *supra.*

Chymotrypsin assays in the presence and absence of proteinase inhibitors

Preparation of gut extracts

Fourth-instar larvae were killed using ethyl-acetate prior the removal of individual gut which were then homogenized in a mortar and pestle in an equal amount [(w/v)] of 50 mM Tris-HCl, pH 8.0 containing 100 μM benzamidine. Insoluble material was removed by centrifugation at 20,000 rpm for 15 min at 4°C and total protein concentration determined using the Bradford method (Bradford, *Anal Biochem 72:* 248-254, 1976) with reagents from BioRad and BSA as a standard.

Assays

Unfractionated gut extract containing approximately 1 μg of buffer soluble protein was added to 10 μL CAPS buffer (0.5 M, 3-[cyclohexylamino]-1-propane-sulfonic acid, pH 10) and made to a final volume of 100 μL in individual wells of a 96 well microtiter plate. Proteinase inhibitors were added over a range of concentrations and incubated for 15 min at 25°C. Bovine chymotrypsin (100 ng) was used as a positive control, both for activity and inhibition. The chromogenic artificial substrate SAAPF*p*NA (N-Succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine 4-nitroanilide) was then added to a final concentration of 1 mM and hydrolysis of the substrate was measured at 405 nm using the SpectraMax 250 microtiter plate reader (Molecular Devices).

Proteinase inhibitors

Lima bean trypsin inhibitor (LBTI), soybean trypsin inhibitor (SBTI), soybean Bowman-Birk inhibitor (SBBI) and chymostatin were all purchased from Sigma-Aldrich Pty.Ltd. Potato Inhibitor I was purchased from Calbiochem-Novabiochem or purified from potato

tubers (Example 6). A crude mixture of Potato inhibitor I and II was also obtained from potatoes. *N. alata* proteinase inhibitors (NaPIs) were purified as described by Atkinson *et al.*, 1993, *supra* and Heath *et al.*, 1995, *supra.* The chymotrypsin inhibitor C1 was purified from bacterial expression cultures. The purity of the inhibitors was assessed by SDS-PAGE and silver staining.

EXAMPLE 2

Isolation of NAPI sensitive and insensitive chymotrypsins from H. punctigera gut

Most published work on *Helicoverpa* chymotrypsins has focused on cDNA clones or the measurement of enzyme activity in unfractionated gut extracts. There are few reports on purification of chymotrypsins from *Helicoverpa* or other lepidopteran species. Johnston and coworkers (1995, *supra*) described partial purification of chymotrypsins from *H.armigera* that employed ion exchange techniques. Peterson and coworkers (*Insect Biochem. Mol. Biol. 25:* 765-774, 1995) purified a chymotrysin from the midgut of the lepidopteran *Manduca sexta* by affinity chromatography on tryptophan methyl ester and Valiatis *et al., Insect Biochemistry and Molecular Biology 29:* 405-415, 1999 used immobilized potato proteinase inhibitor I (PotI) to isolate a chymotrypsin from the Western Spruce budworm. No one has described a procedure that separates individual chymotrypsin isozymes from one another and there is no description of the isolation of two chymotrypsins from a single species where one isozyme is inhibitable by a certain proteinase inhibitor while another is not inhibitable.

Preparation of pure enzymes and N-terminal sequencing

The midgut was dissected from 80 fourth instar larvae and buffer soluble extracts were prepared. The gut extract was depleted of trypsins by repeated passage through a benzamidine-Sepharose affinity column. The unbound protein was collected and applied to an affinity column composed of the immobilized chymotrypsin inhibitor C1 (Figure 1) that had been produced using a bacterial expression system. This column was expected to specifically bind NaPI-sensitive chymotrysins. Proteins that did not bind to this column were applied to a third affinity column composed of either immobilized Potato Type I

(PotI) and Type II inhibitors (PotII) or chymostatin. This column was designed to capture the chymotrypsins that did not bind to the C1 column, that is, the NaPI-insensitive chymotrypsins. Proteins that bound to the affinity columns were eluted with 8 M urea and were subjected to electrophoresis through an SDS-polyacrylamide gel before transfer to a PVDF membrane for N-terminal sequencing (Figures 4 and 5).

About 30 amino acids of N-terminal sequence were obtained that confirmed that the proteins were indeed chymotrypsins, and that the sensitive and insensitive chymotrypsins were products of different genes (Figures 4 and 5).

Preparation of NaPI-insensitive chymotrypsin depleted of trypsins and NaPI sensitive chymotrypsins for biochemical analysis

The midgut was dissected from 100 fourth instar larvae and buffer soluble extracts were prepared. The gut extract was depleted of NaPI-sensitive proteases by passage through an affinity column composed of immobilized NaPI protein (C1, C2, T1-T4). All trypsins and NaPI-sensitive chymotrypsins bound to the column and the NaPI-insensitive chymotrypsin was unbound. This preparation of unbound material was used to study the pH optimum, substrate preference and effect of a range of proteinase inhibitors on the activity of the NaPI-insensitive proteinase. The effect of pH on activity of the insensitive chymotrypsins is illustrated in Figure 6. The enzyme is inactive below pH6 and is most active at pH10-12 consistent with its role in the alkaline midgut of larvae. The best substrate for enzyme assays was determined using seven different commercial substrates. The best substrate was N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sAAPF-pNA) followed by N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide (sAAPL-pNA) and N-methoxysuccinyl-Ala-Ala-Pro-Met-p-nitroanilide (mAAPM-pNA). N-succinyl-Ala-Ala-Ala-p-nitroanilide (sAAA-pNA), benzoyl-tyr- p-NA, Ac-Pro-Leu-Ser-p-NA and Ac-Asn-Gly-Ile-Pro-p-NA were not substrates.

Several proteinase inhibitors were tested for their ability to inhibit the activity of the insensitive chymotrypsins. The buffer used for all assays was 500 mM CAPS, 75 mM NaCl, 2.5 mM $MgCl_2$ at pH 10. The inhibitors were preincubated with 100 ng of bovine

chymotrypsin or the amount of the gut chymotrypsin required to produce the same absorbance as 100 ng of bovine chymotrypsin (note that the *Helicoverpa* enzyme has been depleted of trypsins and sensitive chymotrypsins, but still contains other gut proteins) for 30 min at 30°C before the addition of substrate. The incubation was continued for a further 30 min at room temperature before absorbance was measured at 405 nm. PotI was the best of the proteinaceous inhibitors and NaPI did not inhibit (Table 3).

Experimental protocols

Benzamidine Column

Benzamidine-agarose (1 mL; was purchased from ICN Biomedicals) and contained 35 umoles benzamidine per ml of gel.

Production of the C1-affinity column

DNA encoding the C1 domain of NaPI (Figure 1, Atkinson *et al.*, 1993, *supra*) was amplified from the pNa-PI-2-cDNA (Atkinson *et al.*, 1993, *supra*) using oligonucleotide primers that incorporated *Bam*H1 (5' GACCAGCCGGATCCGATCGGATAT GCACCAAC) [SEQ ID NO:7] and *Hin*dIII (3' GGAGCCAAGCCAAGCTTTGAACGCG GGCAAACTC) [SEQ ID NO:8] sites for cloning into a pQE expression vector (Qiagen). The PCR product was sub-cloned into the pCR (registered trademark) 2.1-TOPO vector (Invitrogen) then excised with *Bam*H1 and *Hin*dIII and ligated into the pQE-30 vector. The expression vector incorporated a hexahistidine tag at the N-terminus of the expressed protein for metal affinity purification. The C1/pQE-30 construct was transformed into the chemically competent *E. coli* strain M15 (Qiagen) prepared according to the method of Inoue *et al., Gene 96:* 23-28, 1990. Bacterial expression cultures were grown and induced according to the procedures outlined in the QiaExpress manual (Qiagen). Expression of the 6H.C1 recombinant protein was achieved by induction with 1 mM of IPTG. Samples (1 ml) were removed from the culture at hourly intervals, collected by centrifugation and resuspended in 1X SDS loading buffer (100 μL) for SDS-PAGE analysis.

Recombinant C1 inhibitor was purified under denaturing conditions using Talon Metal

Affinity Resin (2 mL) (BD Biosciences Clontech) according to the manufacturer's protocol. C1 was eluted from the affinity matrix and examined using SDS-PAGE to confirm purity. Subsequent rounds of expression were conducted under the same conditions and C1 from a total of 2L of culture was purified using the Talon resin. After washing to remove unbound proteins the C1 inhibitor remained bound to the Talon resin which was then used as the affinity column to purify chymotrypsins from gut preparations.

Potato Inhibitor I and potato Inhibitor II affinity column

Cyanogen bromide-activated Sepharose 4B (1 g) was swollen and washed according to the manufacturer's protocol. A mixture of potato I and II Inhibitors (10 mg) was dissolved in coupling buffer [0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3], combined with the gel suspension and incubated over night at 4°C in an end-over-end mixer. The gel was rinsed several times in blocking buffer [0.1 M Tris-HCl, pH 8.0] to remove excess ligand. Following the washes the conjugated Sepharose was mixed with fresh blocking buffer and incubated overnight at 4°C. The gel slurry was transferred to a column (Amersham Biosciences) and washed alternately (x3) with five column volumes of coupling buffer then five column volumes of rinse buffer [0.1 M NaOAc, 0.5 M NaCl, pH 4.0]. Finally the matrix was extensively washed with coupling buffer and stored in 20% (v/v) ethanol at 4°C.

Chymostatin affinity column

Chymostatin (Sigma-Aldrich) was immobilized on EAH Sepharose-4B (1.5 ml; Sigma-Aldrich) according to the manufacturer's instructions. Chymostatin (10 mg) was dissolved in 500 μL of glacial acetic acid then 1 mL of distilled water was added and the pH adjusted to 4.5 with dilute NaOH. The gel suspension and chymostatin solution were combined before EDC [N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide] was slowly added to a final concentration of 0.1 M. The gel suspension was mixed overnight at 4°C before the gel was washed with Milli-Q filtered water to remove excess ligand and unreacted carbodiimide and the gel matrix was stored at 4°C in 20% [(v/v)] ethanol.

Purification of NaPI- sensitive and insensitive chymotrypsins

Eighty gut from fifth instar larvae that had been stored at -80°C were homogenized using a mortar and pestle in 10 ml of gut extraction buffer (20 mM CAPS pH 10, 350 mM NaCl). The gut extract was centrifuged at 15,000 g for 15 min at 4°C and the supernatant was filtered through a syringe filter (0.45 μM; Millipore) then briefly stored on ice before application to the benzamidine affinity column.

The filtered gut extract was passed through the benzamidine column five times to remove the trypsins. The unbound fraction (10 mL) was passed through the C1 affinity column (x3) before the column was washed with 20mL of extraction buffer and bound proteins were eluted with 8 M urea, pH 8.0 (5 mL). Proteins that did not bind to the C1 column were applied to the Potato Inhibitor I and II affinity column prior to washing with 10 column volumes of buffer [20 mM CAPS pH 10, 0.5 M NaCl] and elution of bound proteins with 8 M urea, pH 8.0 (5 mL).

Analysis of affinity purified gut proteins: SDS-PAGE

Samples of gut proteins (5-10 μg) were concentrated using TCA precipitation and resuspended in 0.2 M NaOH (10 μL). SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.1% (w/v)] bromophenol blue) was added and samples were heated to 100°C for 5min prior to separation on 12.5% (w/v) reducing polyacrylamide gels using the MiniProtean II Electrophoresis apparatus (Bio-Rad) at 200 Volts. Broad range or peptide molecular size markers (Bio-Rad) were used to estimate relative molecular masses. Following electrophoresis the proteins were visualized by staining with Coomassie Brilliant Blue R-250 (0.1% (w/v) in 40% (v/v) methanol, 10% (v/v) acetic acid) for 60 min followed by destaining in 40% (v/v) methanol, 10% (v/v) acetic acid or were transferred to either nitrocellulose (0.22 μM pore size; Micron Separations Inc.) or Sequi-Blot Polyvinylidene Fluoride membrane (PVDF; Bio-Rad).

Immunoblotting and N-terminal sequencing

After electrophoresis gels were equilibrated in transfer buffer (192 mM glycine, 48 mM Tris-base, 20% (v/v) methanol) for 10 min prior to the transfer of proteins to a nitrocellulose membrane (0.22 μM pore size; Micron Separations Inc.) using the Mini Trans-Blot apparatus (BioRad) at 100 V for 60 min. Membranes were briefly washed in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) then stained with amido black (1:50 dilution of 0.1% (w/v) amido black, 40% (v/v) methanol, 10% (v/v) acetic acid) to confirm transfer of the proteins and to visualize the molecular size markers. Blots were then blocked by incubation with 3% (w/v) skim milk powder (Dutchjug) in TBST [0.1% (w/v) Tween-20 in TBS] for 1 h at RT, followed by a 1 h incubation with the α-chymotrypsin antibody (HpCH2B; 1:5000 dilution in 3% (w/v) skim milk powder in TBST). The nitrocellulose blots were then rinsed three times (5 min) in TBST and incubated for 1 hour at RT with the secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:5000 in TBST; Amersham Biosciences). Membranes were washed three times (5 min) with TBST and immunoreactive proteins were visualized with Enhanced Chemiluminescence (ECL) reagents and Hyperfilm ECL X-ray film (Amersham Biosciences) according to the manufacturer's instructions.

To obtain N-terminal sequence, samples were transferred to Sequi-Blot PVDF membrane (BioRad) equilibrated in electroblotting buffer (10 mM CAPS, 10% (v/v) methanol, 0.01% (w/v) SDS, pH 11) using the Mini Trans-Blot cell (BioRad) at 100 V for 45 minutes. Following transfer, the membrane was briefly rinsed in Milli-Q water, stained with Coomassie Brilliant Blue (0.1% Coomassie Blue R-250, 1% (v/v) acetic acid, 40% (v/v) methanol) and then destained (50% (v/v) methanol). Finally the membrane was rinsed with water and dried before excision of the appropriate proteins for N- terminal sequencing.

EXAMPLE 3

Cloning cDNAs encoding gut chymotrypsins from H. punctigera

cDNAs encoding the *H. punctigera* chymotrypsins were obtained using two approaches. Both approaches employed PCR amplification of cDNA produced from midgut mRNA extracted from *H. punctigera* larvae at the late fourth and early fifth instar stage of development.

Isolation of chymotrypsin clones using oligonucleotides complimentary to highly conserved regions in H.armigera chymotrypsins

Bown and colleagues (1997, *supra*) have described several cDNA clones encoding *H.armigera* chymotrypsins. Figure 7 shows the predicted proteins and the regions complementary to the oligonucleotides (Figure 8) chosen for PCR amplification of chymotrypsin cDNAs from *H. punctigera*. The PCR products were cloned and sequenced, and five distinct chymotrypsin sequences were obtained (F1Apcr, F1Bpcr, F2Bpcr, F3pcr and F4pcr, Figure 9). These PCR products were used to screen a cDNA library prepared from midgut mRNA isolated from late fourth instar and early fifth instar larvae. Seven distinct cDNA clones were isolated encoding chymotrypsins that were divided into four families based on sequence identity (Figure 10, Table 2). These chymotrypsins share high sequence identity with the *H. armigera* chymotrysins described by Bown *et al.*, 1997, *supra* and with the small number of chymotrypsin sequences reported for *H. virescens* and *H. zea* (Table 6). The chymotrypsins encoded by the full length clones presented in Figure 10 characteristically encode zymogens of approximately 292-295 amino acids, including putative amino-terminal signal peptides of 16-17 residues predicted by the signal peptide prediction program PSORTII. The presence of the residues IVGG (positions 62-65) at the N-terminus of several active chymotrypsins results in the prediction of activation peptides ranging from 35-44 residues in length on the zymogens predicted from the cDNA clones. Furthermore these activation peptides consistently have the dipeptide arginine-isoleucine at their C-termini suggesting a role for trypsin in activating the chymotrypsins. The sequence identities for the mature domains of each translated protein are presented in Table 2. Family 4 was most divergent with less than 20% identity with the other families, while

families 2 and 3 shared most similarity with scores of about 85%. The N-terminal sequence obtained from the NaPI inhibitable chymotrypsin (Figure 4) matched the N-terminal sequence predicted from the cDNA clones encoding chymotrypsins from family 2. The N-terminal sequence of the NaPI -insensitive chymotrypsins was not represented in the 4 families of chymotrypsins represented by the cDNA clones.

TABLE 5

Protein sequence identity (%) betweeen members of the H. punctigera chymotrypsin gene family (mature chymotrypsin domain only)

H. punctigera **chymotrypsin families**	***HpCh1AI***	***HpCh1BI***	***HpCh2A***	***HpCh2B***	***HpCh3A***	***HpCh3B***	***HpCh4AI***	***HpCh5***
HpCh1AI		90	54	53	58	59	<20	57
HpCh1BI			51	51	56	56	<20	55
HpCh2A				94	83	87	<20	73
HpCh2B					82	82	<20	72
HpCh3A						92	<20	70
HpCh3B							<20	72
HpCh4AI								<20

TABLE 6

H. punctigera chymotrypsins are closely related to chymotrypsins from other Helicoverpa species

Helicoverpa punctigera **chymotrypsins**	**GenBank accession**	**Species**	**% amino acid sequence identity**
HpCh1AI	Y12273 AF237417	*Helicoverpa. armigera* *Heliothis virescens*	92 86
HpCh1BI	Y12273 AF237417	*H. armigera* *H. virescens*	91 88
HpCh2A	Y12287 Y12280 Y12281 AF233734	*H. armigera* *H. armigera* *H. armigera* *H. zea*	97 95 95 94
HpCh2B	Y12287 Y12280 Y12281	*H. armigera* *H. armigera* *H. armigera*	92 91 90
HpCh3	Y12279 Y12287	*H. armigera* *H. armigera*	96 82
HpCh4I	Y12272	*H. armigera*	89
HpCh5	AAO75039 Y12281	*Spodoptera frugiperda* *H. armigera*	80 74

Protein sequences most similar to *H. punctigera* were obtained using the BLAST search engine. Genbank accession numbers are listed. The mature activated proteins were compared and percentage of protein sequence identity determined.

The N-terminal region of the insensitive chymotrypsins had two unique stretches of sequence, designated F1 and F2 that were used to design oligonucleotides for PCR amplification of DNA encoding one of the insensitive chymotrypsins (Figure 11) A 641 bp fragment of DNA was obtained with the F1 oligonucleotide that encompassed most of the protein-coding region. This PCR product was used to screen for a full length clone in the *H. punctigera* midgut library. Approximately 0.2% of the 50,000 plaques screened hybridized strongly to the PCR product. Ten plaques were selected and and a full length clone was identified and sequenced (Figure 12). The 921 bp clone had an open reading frame of 828 bp and residues 41-76 of the deduced protein were an identical match to the N-terminal sequence obtained from the purified protein (Figure 12). The clone lacked the

5'signal peptide sequence, but comparison to the other chymotrypsin clones indicated the activation peptide was full length. The active enzyme is predicted to be 236aa in length, with a mass of 24.2 kDa. Protein sequence homology was determined by comparing the mature domains of each of the *H. punctigera* cDNAs. Family 2 and family 3 were most similar to HpCh5 (Rech1a), with about 72 and 70% identity respectively. Family 1 members shared 53-57% identity while Family 4 had about 20% identity with HpCh5. Comprehensive Blast searches at the NCBI facility of both translated and protein databases produced no significant matches. The insensitive chymotrypsin belongs to a new family of *Helicoverpa chymotrypsins* designated Family 5 (Figure 13). A blast search performed on a *Helicoverpa armigera* midgut EST database matched a clone with over 97% sequence identity and 100% sequence similarity Figure 14.

Bovine chymotrypsins A and B and the NaPI-sensitive chymotrypsin (HpCh2A) were compared to the insensitive chymotrypsin (HpCh5) to identify regions of variability that may be involved in low affinity binding to C1 (Figure 15).

Ten substitutions identified in the alignment did not appear to fall into functionally significant regions, whereas the eleventh substitution was associated with one of the β-strands that forms a wall of the primary substrate-binding pocket. The location of this substitution and conversion to an arginine is highly unusual for the S1 domain that is predominantly lined with non-polar residues that define chymotrypsin specificity.

Experimental protocols

Preparation of RNA

Fifty late fourth and early fifth instar larvae were cold anaesthetized before the gut was removed and snap frozen in liquid nitrogen. The 50 gut were ground to a fine powder in the presence of liquid N_2 using a mortar and pestle and were stored at -70°C. The gut tissue (100 mg) was added to 1 mL of TRIZOL (trademark) reagent (Life Technologies) and total RNA was purified and quantified according to the manufacturer's protocol.

RT-PCR amplification of chymotrypsin genes

Chymotrypsin genes from *Helicoverpa armigera* were aligned using ClustalW and regions of high conservation were identified for design of chymotrypsin specific primers. Primers were individually designed to the *H. armigera* clone (CAA72951) due to the high level of divergence to the other chymotrypsin sequences. Forward primers were used in combination with RVG4 to amplify the gene fragments using reverse transcriptase polymerase chain reaction (RT-PCR; Superscript Preamplification System, Gibco BRL) with the protocols supplied. The PCR products were gel purified (Qiagen gel extraction kit), sub-cloned into TOPO PCR2.1 TA cloning vector (Invitrogen) and transformed into chemically competent *E. coli* XL1-Blue cells (Stratagene) prepared according to the method of (Inoue *et al.*, 1990, *supra*). Plasmid DNA was prepared using the QIAprep (registered trademark) Spin Miniprep Kit (Qiagen).

Production and screening of a H. punctigera gut cDNA library:

Library preparation

PolyA+ mRNA was isolated from the RNA (1.0 mg) using conventional protocols. A biotinylated oligonucleotide (dT) primer that binds to the polyA tail tags the transcripts that are subsequently captured on streptavidin paramagnetic particles (PolyATract (registered trademark), mRNA Isolation Systems, Promega). Purified mRNA (5 μg) was used to construct a cDNA library with the Lambda ZAP-cDNA synthesis kit and the Zap-cDNA GigapackIII Gold packaging extract (Stratagene) following the manufacturer's instructions. The amplified library titre was 2.8 x 10^{10} pfu/mL.

Labeling probes

RT-PCR products (50 ng) were individually labelled with [α-^{32}P] dCTP (Amersham Life Sciences) using the MEGAPRIME (trademark) DNA labeling system labeling (Amersham Life Sciences). Unincorporated radiolabeled nucleotides were removed using the Micro Bio-spin P-30 chromatography columns (BioRad) according to the manufacturer's protocol. The double stranded labelled probes were denatured by boiling (5 min), cooled on ice, and then added to the hybridization solution (as below).

Screening the cDNA library

The primary screen was performed using ten (15 cm) Petri dishes per probe, with about 50,000 phage per plate. Preparation of the plates for subsequent plaque lifts and treatment of the membranes prior to hybridization was performed according to instructions provided with the Lambda Zap cDNA synthesis kit (Stratagene). The membranes (Hybond-N; Amersham Biosciences) were prehybridized in 50 ml of 2 x PIPES (0.8 M NaCl, 16 mM Piperazine-1,4-bis(2-ethanesulphonic acid) pH 6.5) 50% (v/v) formamide, 0.5% (w/v) SDS and 100 μg/mL Herring sperm DNA (Boehringer Manningham) at 42°C for 3 hours. Following prehyridization, the incubation solution was replaced with 50 ml of 50% (v/v) formamide, 2 x PIPES buffer, 0.5% (w/v) SDS and 100 μg/mL denatured herring sperm DNA containing the labeled probe and left overnight at 42°C. The hybridization membranes were washed three times in 2 X SSPE/0.1% (w/v) SDS at room temperature and twice in 0.2 X SSPE/0.1% (w/v) SDS at room temperature, before they were blotted on 3 mm Whatman paper and exposed to X-ray film (Kodak XAR-5) for 48 hours at -70°C with intensifying screens.

Due to crowding and hence overlapping between positive clones and non-specific plaques a secondary screen was conducted to isolate individual clones. Two 8.5 cm plates each with 50 plaques were used for each probe. The secondary screen was conducted as described for the primary screen.

At least 50 positive plaques of varying intensities were selected from each screen probed with an individual RT-PCR product. Each plaque was transferred to a 1.5 mL microfuge tube containing 1 mL of SM buffer (0.1 M NaCl, 8 mM $MgS0_4.7H_20$, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin) and chloroform (20 μL) and stored at 4°C. Initially 10 plaques for each probe were excised and converted into pBluescriptII SK(-) phagemids using the ExAssist/SOLR system (Stratagene). The excised phagemids were transformed into the chemically competent *E. coli* strain XL1-Blue cells and plasmid DNA was prepared and sequenced.

DNA sequencing and analysis

The cDNA clones were grouped on the basis of restriction fragment patterns obtained using combinations of the endonucleases *Bam*HI, *Xho*II, *Kpn*I, *Sac*I, *Sac*II, and *Sal*I (Promega). RT-PCR products and cDNA inserts were sequenced in both directions using M13 universal primers at either Micromon sequencing facility at Monash University (Melbourne) or SUPAMAC at the Royal Prince Alfred Hospital in Sydney. The sequence data was edited using the BioEdit v5.0.9.1 software written by Tom Hall, North Carolina State University freely available at the web address: www.mbio.ncsu.edu/BioEdit/bioedit.html. Sequence homologies were assessed using the BLASTN search facility at National Centre for Biotechnology Information (NCBI) and further multiple sequence alignments were performed using ClustalW version 1.4. at the Network Protein Sequence Analysis facility (NPSA; http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl) (Combet *et al.*, *TIBS. 25:* 147-150, 2000).

The web based program 'PSORT II' available at the Human Genome Centre at the University of Tokyo (http://psort.nibb.ac.jp/form2.html), was used to predict signal peptide cleavage points. UTRscan was used to detect functional elements in the 3' untranslated regions of the cDNA clones [Pesole, *Trends Genet, 15:* 378, 1999]. (http://bighost.area.ba.cnr.it/BIG/UTRScan/).

Isolation of a partial cDNA clone encoding a NaPI- insensitive chymotrypsin

A sample of RNA previously purified for the production of the cDNA library was thawed and used as template for the following RT-PCR reaction. Two 5' degenerate primers (5) were designed to unique regions of the N-terminal amino acid sequence and used in combination with the 3' primer RVG4. First strand cDNA synthesis was achieved using the SuperScriptII Preamplification system from Stratagene and was followed by PCR amplification of the target cDNA using the Perkin Elmer thermocycler [25 cycles for 1 min at 94°C, 1 min at 48°C and 1 min at 72°C then 7 mins at 72°C]. PCR products were separated on 1% (w/v) agarose gel (SEAKEM (registered trademark); BioWhittaker Molecular Applications) and a band of approximately 650 bp was excised and purified using the Concert purification system (Gibco). The partial cDNA was cloned into the

TOPO PCR2.1-TA vector (Invitrogen) and transformed into *E. coli* strain XL-BL1 (Stratagene).

Isolation of the insensitive chymotrypsin cDNA clone

The cDNA library prepared from fourth instar larval gut was screened using a partial fragment cloned according to the techniques described above

EXAMPLE 4

Homology modeling of the H. punctigera chymotrypsins

The deduced amino acid sequences from the cDNA clones HpF2B (sensitive) and HpF5 (insensitive) were modeled on the structures of the *Bos taurus* (bovine) and fire ant chymotrypsins, obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank site (http://www.rcsb.org/pdb/). The *Helicoverpa* chymotrypsins are predicted to adopt similar structures to those reported for all the chymotrypsin structures available in the PDB databank. The modeled structure consists of the classic serine protease fold consisting of two, six-stranded anti-parallel β barrels with the catalytic triad located between the two domains. Two surface loops, 60 and 142 are considerably larger in the *H. punctigera* chymotrypsins (Figures 15 and 16). Due to the limitations of modelling, a small amount of ambiguity was present in several surface loops, some of which are cleaved in mammalian chymotrypsins (loop 142), but remain intact within insect chymotrypsins. The only reported crystal structure of an insect chymotrypsin is from the fire ant, *Soenopsis invicta* (Botos *et al., Journal of Molecular Biology 298:* 895-901, 2000) and this was used to help refine the orientation of the surface loops on the model of the *Helicoverpa* chymotrypsin.

C1 was modeled in complex with sensitive and insensitive chymotrypsins to investigate whether substitution of glutamine (or asparagine) 192 (Greer nomenclature, Figure 15) with an arginine would affect the binding capacity of the *Helicoverpa* chymotrypsins. The structure of the chymotrypsin inhibitor (C1) was previously determined by 1H NMR (Nielson *et al.*, 1994, *supra*). No structures of C1 complexes have been solved and

therefore the related proteinase inhibitor PCI-1 from *Solanum tuberosum* in complex with Proteinase B from *Streptomyces griseus* (Greenblatt *et al.*, 1989, *supra*) provided a basis for structural modeling. Figure 17 illustrates the binding region surrounding Gln 192 in the C1-HpF2B chymotrypsin complex. The predicted model of HpF2B and C1 shows that glutamine 192 is not in conflict with any regions on the inhibitor molecule. Comparison to the cognate arginine residue in the insensitive chymotrypsin however suggests there is limited space to accommodate this much larger residue (Figure 18). The modeling viewing program 'Spdbv', predicted Arg 192 to be in direct conflict with threonine 5 of C1. PotI has been identified as a strong inhibitor of the insensitive chymotrypsin from *H. punctigera* and was, therefore, modelled in complex with the insensitive chymotrypsin (Figure 19). Unlike C1, PotI easily accommodates arginine 192 upon binding to the insensitive chymotrypsin.

In summary, the NaPI-insensitive chymotrypsin from *Helicoverpa* species has an arginine in place of an asparagine or glutamine at position 192 that extends into the S1 binding pocket and appears to interfere with C1 binding. Furthermore, it is clear that this arginine residue does not interfere with PotI binding, consistent with the observation that PotI is a much more efficient inhibitor of insect chymotrypsins than the NaPI inhibitors.

EXAMPLE 5

Production and characterization of polyclonal antisera to the NaPI inhibitable and NaPI- insensitive chymotrypsins from H. punctigera

Chymotrypsin clone HpF2B was expressed in *E. coli* fused to a six histidine (6.H) tag at the C-terminus and was purified to homogeneity on Talon metal affinity resin (Figure 20) for injection into a rabbit for production of polyclonal antibodies. N-terminal sequencing of the purified product confirmed the expression of the NaPI inhibitable chymotrypsin (HpCh2B). After the fourth boost with antigen, the serum was collected and tested on protein blots of bacterially expressed protein and unfractionated gut extracts. The antibody detected the full-length recombinant chymotrypsin at a dilution of 1 in 2500 as well as several break-down products. Unfractionated gut extract and a sample of protein bound to

the C1 affinity column were also stained with the anti-HpCh2B antibody which detected the mature native form of the enzyme.

Purified 6H.HpCh2B was used to test the detection limit of anti-HpCh2B antibody by comparison of immunoblots to silver stained SDS-PAGE gels. The antibody detected 20 ng of bacterially expressed chymotrypsinogen and also recognized the mature form of the native chymotrypsin isolated from gut of *H. punctigera*.

The cDNA (HpF5) encoding the NaPI-insensitive chymotrypsin (HpCh5) was expressed in *E. coli* in a similar manner except the six.histidine tag was fused to the N-terminus of the expressed protein. The polyclonal antiserum that was raised against the bacterially expressed NaPI inhibitable chymotrypsin (HpCh2B) did not cross-react with bacterially expressed NaPI-insensitive chymotrypsin (HpCh5) on protein blots (Figure 21). Likewise the antiserum raised against HpCh5 did not bind to HpCh2B. This indicates that these antisera can be used to specifically distinguish between and monitor levels of the NaPI-insensitive and sensitive chymotrypsins in unfractionated gut extracts.

Experimental protocols

Preparation of antigen for immunization

The cDNA clone 'HpF2B'which encodes an NaPI sensitive chymotrypsin (Figure 11) was amplified by polymerase chain reaction (PCR) using two oligonucleotides (Table 7) that incorporated *Nco*I and *Bgl*II restriction sites at the 5' and 3' ends of the cDNA respectively.

TABLE 7

Degenerate primers designed to two unique regions in the N-terminus of the insensitive chymotrypsin protein. Primer positions are shown in the amino acid sequence by matching typeface

N-terminal sequence of NaPI-insensitive chymotrypsin IVGGSLSS**VGQIP**YQAGL*VIDLAGG*QAVCGGSLISA [SEQ ID NO:9]	
Primer Name	**Oligonucleotide sequence 5'-3'**
Fw2ResChy	TC(AGCT) GT(AGCT) GG(AGCT) CA(AG) AT(ACT) CC [SEQ ID NO:10]
FwResChym	GT(AGCT) AT(ACT) GA(CT) CT(AGCT) GC(AGCT) GG(AGCT) GG [SEQ ID NO:11]

TABLE 8

PCR amplification primers for bacterial expression of chymotrypsin HpF2B

Primer Name	**Restriction site**	**Sequence**
5' Hc35PQE-60Fw	*Nco*I	TTA ACC ATG GTG ATC GAC CTC [SEQ ID NO:12]
Hc35PQE-60Rv	*Bgl*II	GAT GAG ATC TGA GAC GTT GGT TG [SEQ ID NO:13]

The amplified region consisted of the pro-peptide and mature domain of the chymotrypsinogen, but lacked the putative secretion signal. Digests using *Nco*I and *Bgl*II enzymes (Promega) were performed on the PCR amplified product and the pQE-60 expression vector (Qiagen). The pQE-60 vector provides a His-tag at the C-terminus of the expressed protein. Each restriction digest was purified using WIZARD (registered trademark) DNA clean up system (Promega) and the vector and chymotrypsin insert were subsequently ligated using standard molecular biology techniques (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 3rd edition 2001) The ligation mix was heated at 65°C for 10 min before being transformed into the *E. coli* strain XL1-Blue. Plasmid DNA was prepared for sequencing and

subsequent transformation into the *E. coli* cell line M15 (Qiagen) using the QIAPREP (registered trademark) Spin Miniprep Kit (Qiagen).

Expression and purification of the recombinant chymotrypsinogen was performed under denaturing conditions according to the methods detailed in the QiaExpress manual (Qiagen). The purity of the expressed chymotrypsinogen was assessed by SDS-PAGE and the identity of the recombinant protein confirmed by N-terminal sequencing. Preparation of the chymotrypsin for injection consisted of removal of the urea by dialysis against 50 mM Tris-HCl pH 8.0. During this process most of the protein aggregated. The aggregated protein was collected by centrifugation and resuspended in 1 mL of 50 mM Tris-HCl, pH 8.0 for injection. The protein concentration was approximated by the comparison of a 10 μL sub-sample to a series of bovine trypsin standards (Sigma) using SDS-PAGE and Coomassie staining.

The cDNA clone 'HpF5' which encodes the NaPI-insensitive chymotrypsin (Figure 12) was PCR amplified essentially as described for clone HpF2B above except the forward primer (FwpMalRECH) incorporated a Histidine tag at the N-terminus of the expressed protein and the reverse primer (RvRECH) contained a stop codon and thus prevented incorporation of a Histidine tag at the C-terminus from the pQE-60 expression vector.

EXAMPLE 6

Bioassays with PotI inhibitor and Helicoverpa larvae

Large quantities of the PotI inhibitor were purified from potato tubers to evaluate the combined effect of NaPI and PotI on the growth of *H.armigera* larvae. Bioassays confirmed that addition of PotI significantly enhances the activity of the NaPI inhibitors. Caterpillars fed NaPI and PotI in combination (0.26 and 0.34% (w/v), respectively) were 34% the size of control larvae at the fifth instar stage of development whereas caterpillars feeding on NaPIs alone were about 84% the size of the controls. Based on these results we decided to clone genes encoding the PotI inhibitors for transfer into cotton plants. Two PotI genes (PotIA and PotIB) were isolated using mRNA isolated from potato tubers and wounded potato leaves. These cDNA clones were used to construct vectors for bacterial expression of the PotI proteins. The bacterially produced PotI proteins totally inhibited the NaPI-insensitive chymotrypsin isolated from *H. punctigera* larvae. Both PotI genes were incorporated into constructs for cotton transformation. Purified PotI inhibitor from potato tubers was used to generate specific antibodies that were used to monitor levels of PotI produced by transgenic plants.

Experimental protocols

Purification of PotI inhibitor from potato tubers

Potato tubers (2 Kg, *Solanum tuberosum* var Russet Burbank) were diced and soaked overnight at 4°C in two litres of 50 mM H_2SO_4. The tissue was homogenized in a blender and insoluble material was removed by filtration through two layers of Miracloth followed by centrifugation (13,000 rpm, 15 min, 4°C). The supernatant was adjusted to pH 7.8 with 10 M NaOH, heated for 30 min in boiling water and cooled before precipitated material was separated by centrifugation (13,000 rpm, 12 min, 4°C). Soluble proteins were precipitated with ammonium sulphate (80% saturation), collected by centrifugation and redissolved in gel filtration buffer (150 mM KCl, 10mM Tris-HCl, pH 8) before they were applied to a Sephadex G75 column (85 x 2.54cm). Elution fractions (50mL) containing PotI were identified using SDS-polyacrylamide gel electrophoresis and inhibition assays

with bovine chymotrypsin. PotI containing fractions were pooled, dialysed and freeze dried. The protein in these pooled fractions was examined by reverse phase -HPLC on a system Gold HPLC (Beckman, Fullerton, CA) coupled to a 166 detector (Beckman). The analytical RP-HPLC was conducted on a Brownlee Aquapore RP300 C8 column (4.6 x 100mm; Perkin-Elmer). The protein was eluted with a linear gradient of 0-100% (v/v) buffer B (60%[v/v] acetonitrile in 0.089% [v/v] trifluoroacetic acid) at a flow rate of 1 mL min-1 over 40min (Figure 22). The identity of the PotI proteins was confirmed by N-terminal sequencing and mass spectrometry and at least two PotI isoforms were identified.

Growth of H. armigera larvae on artificial diet containing NaPI and PotI

PotI protein isolated from potato tubers and NaPI peptides isolated from *N. alata* stigmas, were incorporated into a cotton leaf based artificial diet and fed to *H.armigera* neonates (Figure 23). PotI had an additive affect on larval growth. Larvae fed NaPI only were inhibited in growth by 16% compared to control larvae while larvae fed both NaPI and PotI were inhibited in growth by 75% compared to control larvae (Figure 23).

Isolation of cDNAs encoding Potato proteinase inhibitor 1 (PotI)

Potato proteinase inhibitor I cDNA was synthesized using reverse transcriptase-PCR and total RNA from potato tubers or wounded potato leaves. First strand cDNA was prepared using Thermoscript RT-PCR with Oligo $(dT)_{20}$ primers (Life Technologies). PotI cDNA sequences were subsequently amplified using gene specific primers 5' CGG-GAT-CCA-TGG-AGT-CAA-AGT-TTG-C-3' [SEQ ID NO:14] (sense) and 5'-GCG-TCG-ACG-CTT-AAG-CCA-CCC-TAG-G-3' [SEQ ID NO:15] (antisense) that were designed to anneal to the 5' and 3' ends of the open reading frame of the published PotI genomic sequence M17108 (Cleveland *et al.*, *Plant Mol. Biol. 8:* 199-207, 1987) and included restriction sites *Bam* HI and *Sal* I respectively.

Two PotI homologs were isolated. StPotIA was derived from wounded leaf RNA and StPotIB was derived from tuber RNA. StPotIA and StPotIB cDNA share 92.6% nucleic acid sequence identity and share 86% amino acid sequence identity (Figure 25).

The predicted amino acid sequence and comparison to other members of the Potato Inhibitor I family are presented in Figure 24. StPotIB is very similar to published PotI sequences from potato tuber and shares a methionine at the P1 reactive site. In contrast, StPotIA contains an alanine residue at P1 and also has an additional four amino acids at positions 41 to 44. Additional amino acids in this position have not been reported for other potato PotI isolates although they have been found in a wound induced PotI from tomato.

Expression of StPotIA and StPotIB in E. coli

DNA encoding PotI without the endoplasmic reticulum signal sequence (amino acids 37-111 in StPotIA and 37-107 in StPotIB) was amplified by PCR. Primers used were 5'-CGG-GAT-CCA-AGG-AAT-CGG-AAT-CTG-3' [SEQ ID NO:16] (StPotIA sense), 5'-CGG-GAT-CCA-AGG-AAT-TTG-AAT-GC-3' [SEQ ID NO:17] (StPotIB sense) and 5'-CGA-GCT-CTT-AAG-CCA-CCC-TAG-G-3' [SEQ ID NO:18] (StPotIA/B antisense). PCR products were initially cloned into the pGEM T-Easy vector (Promega) before they were excised with *Bam*HI and *Sac*I and ligated into the bacterial expression vector pQE30 (Qiagen) which provides a 6x His-tag at the N-terminus of the expressed protein.

The His-tagged PotI proteins were expressed in *E. Coli* (BR21 DE3 Codòn Plus strain (Stratagene) for StPotIA and M15 strain (Qiagen) for StPotIB). Cells were induced with 1 mM IPTG, harvested by centrifugation and lysed in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0. Cell debris was removed by centrifugation at 10,000 g for 5 min and the His-tagged PotI was purified from the supernatant by metal-affinity chromatography on Talon resin (Clontech). Bound protein was eluted from the resin with 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.0 and elution was monitored by SDS-PAGE. The StPotIA and StPotIB proteins were purified further by RP-HPLC (Figure 25) their identity was confirmed by N-terminal sequencing and mass spectrometry.

Inhibition of the NaPI-insensitive chymotrypsins from H. punctigera by StPotIA and StPotIB

The inhibitory activity of StPotIA and StPotIB against the NaPI-insensitive chymotrypsins from *H punctigera* was determined by preincubating the NaPI-insensitive protease (10 µL) with varying amounts of StPotIA and StPotIB (0-600 nM) in 133 mM CAPS buffer, pH 10.0 at 30°C in 96 well microtitre plates. After the 30 min preincubation, the incubation was started by the addition of substrate (SA_2PFpNA, SA_2PLpNA or SA_2PMpNA) to a final concentration of 1 mM in a final volume of 100 µL. Absorbance was measured at 405 nm after 30 or 60 min.

StPotIA, StPotIB and the mix of PotI isoforms isolated from potato tuber inhibited the NaPI-insensitive chymotrypsin in the *Helicoverpa punctigera* gut extract (Figure 26). At least 75% of SA_2PFpNA or SA_2PLpNA hydrolysis by the NaPI-insensitive chymotrypsin was inhibited by the addition of 300 nM of StPotIA, StPotIB or a mix of PotI isoforms isolated from tuber. Inhibition of SA_2PMpNA hydrolysis was lower (40%).

Production of transgenic cotton expressing NaPI and StPotIA

Two gene constructs (pHEX2 and pHEX6) were prepared for transformation of cotton (*Gossypium hirsutum*). pHEX2 consists of a 35S promoter driving the NaPI gene with a 35S terminator, inserted into the binary vector pBIN 19 (Bevan, *Nucl. Acids Research, 12:* 8711-8721, 1984). pHEX6 consists of a 35S promoter driving the StPotIA gene with a 35S terminator inserted into the binary vector pBIN 19.

Transgenic cotton was produced using the method of Umbek et al., *Biotechnology, 5:* 263-266, 1987) with modifications. Hypocotyl sections of cotton Cv Coker 315 were co-cultivated with *Agrobacterium tumefaciens* strain LBA 4404 containing the required binary vector. Callus was induced on media consisting of MS salts, B5 vitamins, 3% glucose, 0.9 g/L $MgCl_2$ (hexahydrate), 1.9 g/L potassium nitrate, 2 g/L Gelrite, 0.1 mg/L Kinetin, 0.1 mg/L 2,4-D, 500 mg/L carbenicillin, 35 mg/L Kanamycin. Embryogenic callus was induced by growing the callus on the same media but without hormones. Embryos were excised and incubated on media in petri dishes (Stewart and Hsu, *Planta*

137: 113-117, 1977). Germinated embryos that had produced roots and true leaves were transferred to containers for further development and then transferred to soil and grown in a growth cabinet at 27°C.

Production of transgenic cotton expressing NaPI and StPotIA

To produce plants expressing both genes, pollen from a transgenic line expressing NaPI was used to pollinate a flower from a plant expressing StPotIA and the seed collected. One progeny plant (plant 3) was identified as expressing both genes by immunoblot analysis.

Leaves from plant 3 were used in a bioassay with *H. armigera* (Figure 27). While expression of either NaPI or StPotIA in the leaves only resulted in a small inhibition of larval growth compared to the control, expression of both proteins had a synergistic effect on larval growth.

EXAMPLE 7

Baculovirus expression of HpCh5

Addition of the signal peptide sequence to HpCh5

While the HpF5 clone encoded the entire chymotrypsinogen sequence of HpCh5 with the activation domain it did not encode the signal peptide required for correct targeting of the protein to the endoplasmic reticulum (ER). For baculovirus expression, an ER signal sequence was added to the HpF5cDNA using two overlapping oligonucleotides corresponding to the 19 amino acids of the ER signal peptide from HpCh2A (Figure 10). The ER signal sequence was added to the preactivation and mature domains of HpCh5 in a two-step PCR (Table 9).

TABLE 9

PCR amplification primers for addition of an ER signal sequence to HpCh5

Primer	Sequence
FWBacRECH1 (5'-3')	TTG GCT TTC GCC GCG GTC GTC TCC GCG AGG AAC GGG TCC C [SEQ ID NO:19]
FWBacRECH2 (5'-3')	GGA TCC ATG AAA CTC TTG GCT GTG ACT CTA TTG GCT TTC G [SEQ ID NO:20]
RvRECH (3'-5')	G ATC AAC GGC CAG CTC TAA AAG CTT [SEQ ID NO:21]

The first PCR used primers FwBacRECH1 and RvRECH with the HpF5 cDNA template to add the first half of the ER signal sequence. The second PCR used FwBacRECH2 together with RvRECH and the product of the first PCR reaction as template. At each step, the amplification products were purified after electrophoresis on 0.7% (w/v) agarose gels before they were used for subsequent PCRs.

Cloning of HpF5/ER into pFastBac vector

The HpF5 cDNA with the ER signal sequence (HpF5/ER) was subcloned into the pCR (registered trademark)-2.1 TOPO vector (Invitrogen) and was sequenced at the Micromon, DNA sequencing facility, Monash University, Victoria, Australia. Recombinants with the correct sequence were digested with *Eco*RI and gel purified before they were digested with *Bam*HI and *Hin*dIII and ligated into the pFastBac vector (Invitrogen) and transformed into *E. coli* XL1 Blue cells.

Transposition of pFastBac/HpF5/ER construct into E. coli DH10Bac cells

E. coli XL1 Blue cells were screened for the presence of the HpF5/ER cDNA in the pFastBac vector (pFastBac/HpF5/ER) by PCR and restriction digest. Minipreps were performed on positive transformants. *E. coli* DH10Bac competent cells containing bacmid DNA and the helper plasmid required for transposition of HpF5/ER to the bacmid DNA were thawed on ice. Approximately 1 ng of pFastBac/HpF5/ER recombinant plasmid was added to the cells (150 μL) and after gently mixing the mixture was transferred to a pre-chilled GENE PULSER (registered trademark)/*E. COLI* (trademark) pulser cuvette. The

cuvette was placed in the electroporation apparatus (BioRad) and a pulse of 1.7 Amps was applied. LB (1 mL) was then added and the sample was transferred to a 10 mL capped tube and allowed to recover on a shaking incubator (190 rpm) at 37°C for 4 hours. A sample was withdrawn and serially diluted (10^{-1}, 10^{-2}, and 10^{-3}) using LB medium before 100 µL of each dilution was spread evenly onto LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentiamicin, 10 µg/mL tetracycline, 100 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 40 µg/mL IPTG prior to incubation for 48 hours at 37°C. A pFastBac vector with no insert was treated the same way and used as a control.

Isolation of recombinant bacmid DNA

Recombination between the pFastBac/HpF5/ER vector and the bacmid DNA was detected by the presence of white colonies as apposed to blue colonies, indicating a disruption in the *lacZα* gene. To confirm the recombination was stable, the colonies were restreaked on selective media and incubated for 24 hours. White colonies were used to inoculate 10 mL LB media containing 50 µg/mL kanamycin, 7 µg/mL gentiamicin and 10 µg/mL tetracycline for isolation of the recombinant bacmid DNA. The same procedure was performed with a blue colony (not recombinant) selected as a control. Cells were grown overnight at 37°C with shaking at 190 rpm. Cultures were centrifuged at 14,000 × *g* for 5 min. The supernatant was discarded and the pellet was resuspend in 0.3 ml of Solution I (15 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/mL RNase A]. Solution II (0.2 N NaOH, 1% (w/v) SDS) was then added and gently mix and incubated at room temperature for 5 min. Potassium acetate (0.3 mL, 3 M, pH 5.5) was slowly added and the samples were placed on ice for 10 min before, they were centrifuged for 10 min at 14,000 × *g* to remove the thick white precipitate. The supernatant was then transferred to a 1.5 mL microfuge tube containing 0.8 ml absolute isopropanol and was mixed by inversion. After 10 min on ice samples were centrifuged for 15 min at 14,000 × *g* at room temperature. The supernatant was removed and pellet was washed twice with 0.5 mL of 70% (v/v) ethanol. The sample was then centrifuged for 5 min at 14,000 × *g* at room temperature before the 70% (v/v) ethanol was removed and the DNA pellet was air dried. The DNA was then dissolved in 60 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20°C until needed. The bacmid was examined by electrophoresis on a 0.5% (w/v) agarose gel

prepared in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8) buffer at 23 V for 12 hours with λ DNA/*Hind*III Fragment markers (MBI Fragments). The bacmid was also screened using PCR with M13 Forward and reverse primers.

Transfection of HIGH FIVE (trademark) cells with recombinant bacmid DNA

Approximately 9×10^5 HIGH FIVE (trademark) insect cells were placed into a 35-mm well in a 6-well plate (NUNCLON (trademark) Δ Surface) in 2 ml of EX-CELL (trademark) 405 media with 50 units/mL penicillin and 50 μg/mL streptomycin (JRH Biosciences). Cells were left at 27°C for 1 hour to allow them to attach to the plate. Two solutions were prepared for each transfection each adding a different amount of bacmid miniprep (5, 10 or 20 μL); Solution A: mini-prep of bacmid DNA into 100 μL EX-CELL (trademark) 405 media without antibiotics, and Solution B: 6 μL of CELLFECTIN (registered trademark) Reagent (Gibco BRL) into 100 μL EX-CELL (trademark) 405 media without antibiotics. Solutions A and B were combined and incubated for 1 hour at room temperature followed by 0.8 mL of selection free media added. After the cells had attached to the plate, they were washed once with 2 mL media without antibiotics. The media was removed from cells and was replaced with the DNA containing solution. Cells were incubated for 5 hours in a 27°C incubator before the transfection mixture was aspirated and replaced with 2 mL of media containing antibiotics. Cells were incubated further at 27°C for up to 72 hours.

After 24, 48 and 72 hours, 2 mL of the supernatant was transferred to a 10 mL sterile capped tube and centrifuged for 5 min at 500 × *g*. The virus-containing supernatant was transferred to a fresh tube and stored at 4°C, protected from light until required.

Infection of insect cells with recombinant baculovirus particles

Two 10 mL cultures were set up for each transfection. Each culture was infected with 100 μl of harvested virus (~2×10^7 pfu/mL). Infected cells were harvested at 24, 48, and 72 hours. These samples were later analyzed for protein expression by immunoblot analysis with the anti-HpCh5 antibody.

EXAMPLE 8

Baculovirus expression of HPCH5

Primer design for ER signal sequence

The original HpF5 cDNA clone did not encode an ER signal sequence. While the ER signal sequence was not required for bacterial expression it was essential for baculovirus expression. An ER signal sequence was thus constructed using the DNA sequence from chymotrypsin family 2A which is most closely related to the chymotrypsin family 5.

The sequence was added using PCR reactions with two primers encoding part of the ER signal sequence. The FwBacRECH1 primer had a silent mutation to remove the *Bam*HI restriction digest site and encoded half the ER signal sequence. The FwBacRECH2 primer encoded the remainder of the ER signal sequence and introduced a *Bam*HI restriction site (Figure 28).

Amplification and ligation of HpCh5 into the pFastBac vector

PCR using primers the FwBacRECH1 and RvRECH and HpF5cDNA as the template yielded a product of ~900 bp. A second PCR using this PCR product together with FwBacRECH2 and RvRECH primers was performed to yield a product of ~930 bp that encoded the HpCh5 protein with an ER signal sequence. The amplified product was subcloned into pCR (registered trademark)-2.1 TOPO vector (Invitrogen) before transfection into TOP10 competent cells. Colonies were screened for the presence of insert using M13 forward and reverse primers. Restriction digests of the isolated plasmids with *Eco*RI then subsequently with *Bam*HI and *Hin*dIII yielded a product of the expected size of ~930 bp.

The ~930 bp fragment was subsequently ligated into the pFastBac vector to create pFastBac/HpF5/ER. The presence of the HpF5 cDNA insert in the pFastBac vector was confirmed by restriction digests with *Bam*HI and *Hin*dIII and PCR using FwBacRECH2 and RVRECH primers (Table 9). Plasmids containing the insert were sent to Micromon for DNA sequencing.

Analysis of Bacmid DNA

The transfection of the recombinant pFastBac vector into DH10Bac cells resulted in the transposition of the HpF5 insert into the bacmid DNA. Recombinant bacmid DNA was isolated from the transfected cells and was separated on a 0.5% (w/v) agarose gel.

The bacmid was checked for the HpF5 cDNA insert by PCR analysis with M13 forward and reverse primers. The expected ~3300 bp fragment was further analysed using the primers to HpF5/ER cDNA and M13 to ensure the insert was in the correct orientation. The positive bacmid DNA was subsequently used for the transfection of insect cells.

Virus formation and production of HpCh5 in the baculovirus expression system

Three different concentrations of bacmid DNA were used to transfect the insect cells for production of baculovirus. After transfection, the cells were incubated at 27°C for 72 hours before the culture medium containing the virus was collected. The medium was tested by immunoblot analysis with the α-HpCh5 antibodies for the production of HpCh5 protein, which indicated virus was being produced (Figure 29A). Many controls were used to monitor the transfection. These included pFastBac vector alone, transfection of DNA from a blue colony, incubation of cells with CELLFECTIN (registered tradmark) alone and non-transfected cells (Figure 29A). The medium containing virus was used in a subsequent experiment to infect more insect cells on a larger scale and a time course was performed over a 72 hour period to monitor the regulation of HpCh5 expression (Figure 29B).

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding a chymotrypsin from *Helicoverpa* spp. or a variant, derivative, homolog or analog of said chymotrypsin, wherein said chymotrypsin exhibits resistance to a proteinase inhibitor (PI) from *Nicotiana alata.*

2. The isolated nucleic acid molecule of Claim 1 wherein the nucleotide sequence encodes an amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least about 75% similarity to SEQ ID NO:2 after optimal alignment.

3. The isolated nucleic acid molecule of Claim 2 wherein the nucleotide sequence encodes an amino acid sequence set forth in SEQ ID NO:2.

4. The isolated nucleic acid molecule of Claim 1 wherein the nucleotide sequence is as set forth in SEQ ID NO:4 or SEQ ID NO:6 or a nucleotide sequence having at least about 75% identity to SEQ ID NO:4 or SEQ ID NO:6 after optimal alignment or a nucleotide sequence capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions.

5. The isolated nucleic acid molecule of Claim 4 comprising a nucleotide sequence as set forth in SEQ ID NO:4.

6. The isolated nucleic acid molecule of Claim 1 encoding a variant of the chymotrypsin wherein the variant is an N-terminal signal sequence comprising an amino acid sequence set forth in SEQ ID NO:3 or an amino acid sequence having at least about 75% similarity to SEQ ID NO:3 after optimal alignment.

7. The isolated nucleic acid molecule of Claim 6 wherein the nucleotide sequence is set forth in SEQ ID NO:5 or a nucleotide sequence having at least 75% identity to SEQ ID NO:5 after optimal alignment or a nucleotide seuence capable of hybridizing to SEQ ID

NO:5 or its complementary form under low stringency conditions.

8. The isolated nucleic acid molecule of Claim 1 encoding a variant of the chymotrypsin wherein the variant comprises an amino acid other than arginine at position 192.

9. The isolated nucleic acid molecule of Claim 8 wherein the variant comprises a glutamine at position 192.

10. A vector comprising a nucleic acid molecule of any one of Claims 1 to 9.

11. The vector of Claim 8 wherein the vector is an expression vector.

12. The vector of Claim 10 wherein the expression vector is operable in a prokaryotic cell.

13. The vector of Claim 11 wherein the experssion vector is operable in a eukaryotic cell.

14. The vector of Claim 13 wherein the eukaryotic cell is an insect cell.

15. The vector of Claim 14 wherein the vector is a baculovirus vector.

16. A genetically modified cell comprising a nucleic acid molecule of any one of Claims 1 to 9.

17. The genetically modified cell of Claim 16 wherein the cell is a prokaryotic cell.

18. The genetically modified cell of Claim 16 wherein the cell is a eukaryotic cell.

19. An isolated chymotrypsin from *Helicoverpa* spp. wherein said chymotrypsin

exhibits resistance to a PI from *N. alata* or a variant, derivative, homolog or analog of said chymotrypsin.

20. The isolated chymotrypsin of Claim 19 comprising an amino acid sequence set forth in SEQ ID NO:2 or an amino acid sequence having at least about 75% similarity to SEQ ID NO:2 after optimal alignment.

21. The isolated chymotrypsin of Claim 20 comprising an amino acid sequence as set forth in SEQ ID NO:2.

22. The isolated chymotrypsin of Claim 19 encoded by a nucleotide sequence having at least about 75% identity to SEQ ID NO:4 or SEQ ID NO:6 after optimal alignment or a nucleotide sequence capable of hybridizing to SEQ ID NO:4 or SEQ ID NO:6 or its complementary form under low stringency conditions.

23. The isolated chymotrypsin of Claim 22 encoded by a nucleotide sequence set forth in SEQ ID NO:2.

24. The isolated chymotrypsin of Claim 19 wherein the variant is an N-terminal signal sequence.

25. The isolated chymotrypsin of Claim 24 wherein the signal sequence comprises an amino acid sequence as set forth in SEQ ID NO:3 or an amino acid sequence having at least 75% similarity thereto after optimal alignment.

26. The isolated chymotrypsin of Claim 19 encoded by a variant comprises an amino acid other than arginine at position 192.

27. The isolated chymotrypsin of Claim 19 encoded by a variant comprises a glutamine at position 192.

28. The isolated chymotrypsin encoded by a nucleic acid molecule of any one of Claims 1 to 9.

29. An antagonist of a chymotrypsin as defined in any one of Claims 19 to 28.

30. The antagonist of Claim 29 wherein the antagonist binds or interacts with the chymotrypsin at or near amino acid residue position 192.

31. The antagonist of Claim 29 or 30 wherein said antagonist is PotI.

32. A composition comprising an antagonist of Claim 29 or 30.

33. An anti-insect composition comprising an antagonist of Claim 29 or 30.

34. A genetically modified plant comprising cells capable of producing an antagonist of a chymotrypsin as defined in any one of Claims 19 to 26 or expression of a nucleic acid molecule as defined in any one of Claims 19 to 26.

35. A genetically modified plant comprising cells capable of producing an antagonist of chymotrypsin-HpCh5 or HpF5 gene expression.

36. The genetically modified plant of Claim 34 or 35 wherein the plant is a monocotyledonous plant.

37. The genetically modified plant of Claim 34 or 35 wherein the plant is a dicotyledonous plant.

38. The genetically modified plant of Claim 34 or 35 wherein the plant is a crop plant, vegetable plant or ornamental plant or tree.

39. The genetically modified plant of Claim 34 or 35 wherein the plant products

PotI.

40. The genetically modified plant of Claim 37 wherein the plant is cotton, sweet corn, tomato, tobacco, piniento, potato, sunflower, citrus, plums, sorghum, leeks, soybean, alfalfa, beans, pidgeon peas, chick peas, artichokes, curcurbits, lettuce, *Dianthus* (an ornamental plant), geraniums, cape gooseberry, maize, flax and linseed, alfalfa, lupins, broad beans, garden peas, peanuts, canola, snapdragons, cherry, sunflower, pot marigolds, *Helichrysum* (an ornamental plant), wheat, barley, oats, triticale, carrots, onions, orchids, roses and petunias.

41. The genetically modified plant of Claim 40 wherein the plant is a cotton plant.

42. The genetically modified plant of any one of Claims 34 to 41 comprising a nucleic acid molecule encoding Pot1A and/or Pot1B.

43. Seeds or other reproduction material from the plant of any one of Claims 34 to 42.

44. A method for modulating activity of the HpCh5 or a homolog or variant thereof in an insect, said method comprising contacting the HpCh5 protein or its homolog or variant with an effective amount of an agent for a time and under conditions sufficient to decrease or increase HpCh5 activity.

45. A method for modulating expression of HpF5 or homolog or variant in an insect, said method comprising contacting HpF5 or its homolog or variant with an effective amount of an agent for a time and under conditions sufficient to decrease or increase HpF5 expression.

46. A method is provided for the isolation of and separation of individual isoforms of chymotrypsin, said method consisting of:

(i) affinity chromatography of insect gut extracts initially with benzamidine sepharose to bind trypsins;

(ii) further affinity chromatography of the unbound proteins using immobilized *N. alata* serine proteinase inhibitor C1 to bind all NaPI inhibitable chymotypsins; and

(iii) affinity chromatography of the eluate from (ii) with immobilized PotI and PotII or chymostatin to bind the remainder. The putative NaPI-insensitive chymotrypsins are then eluted with 8 M urea.

47. A method for screening for an antagonist of a NaPI-insensitive chrymotrypsin from *Helicoverpa spp* said method comprising contacting same chrymotrypsin with a potential antagonist and screening for chrymotrypsin activity.

48. An isolated inhibitor identified by the method of Claim 47.

10/554237

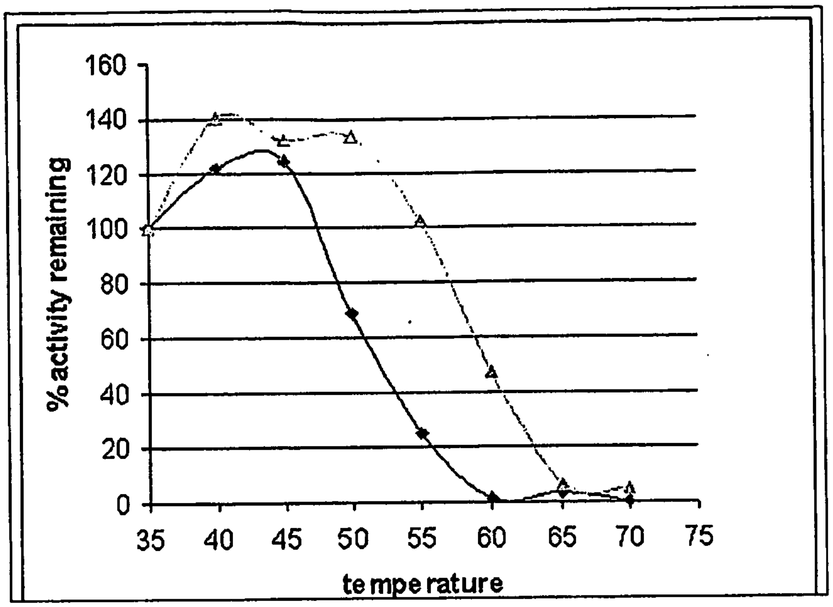


Figure 1

10/554237

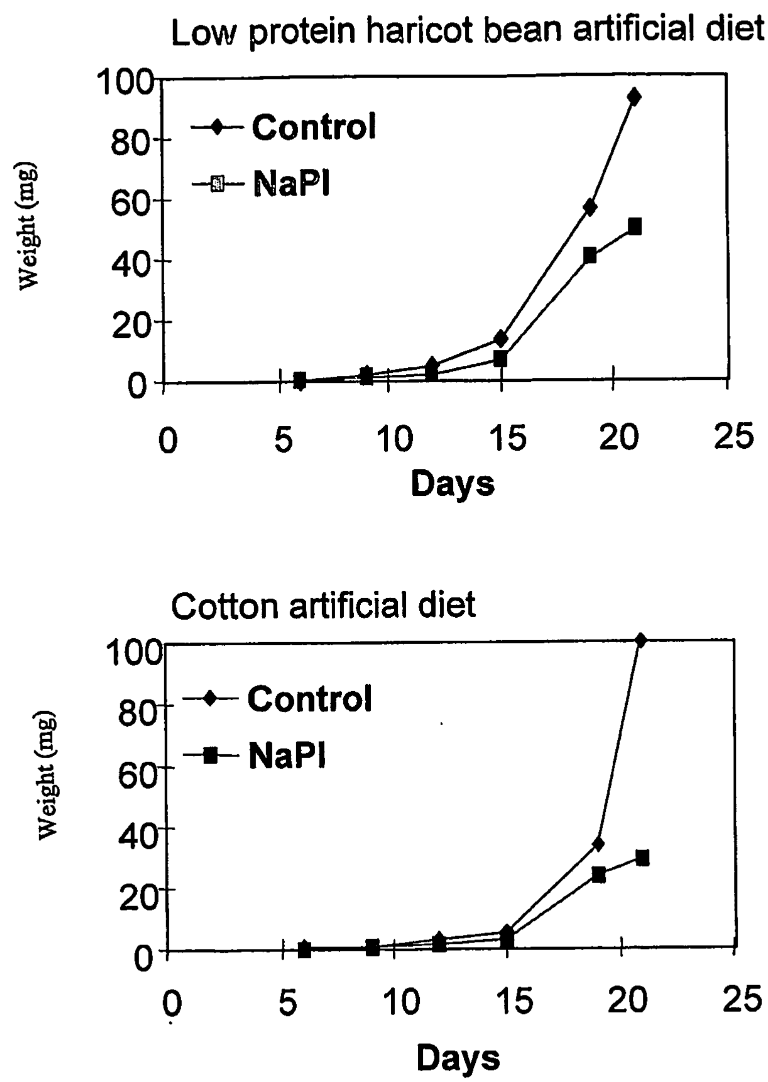


Figure 2A

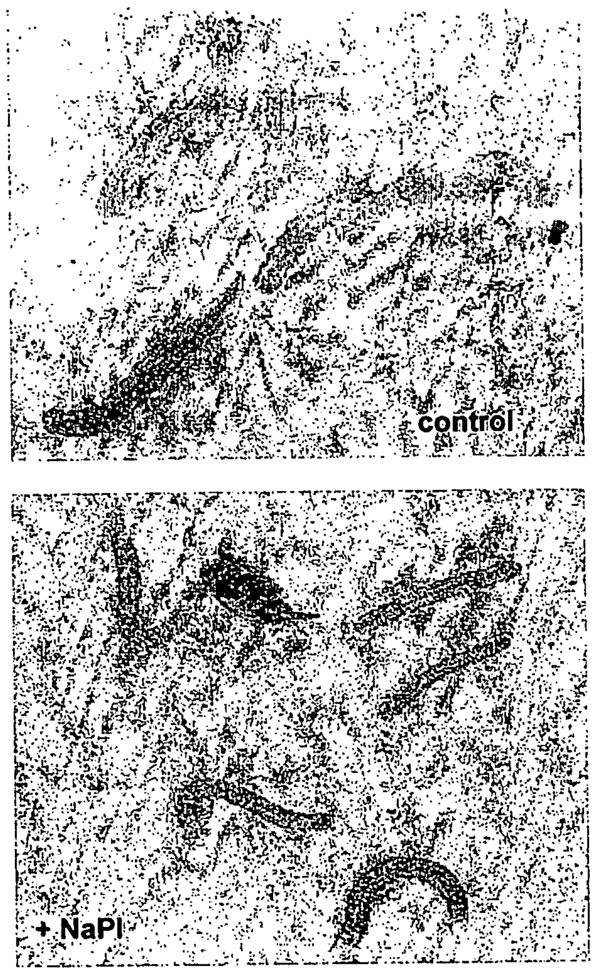
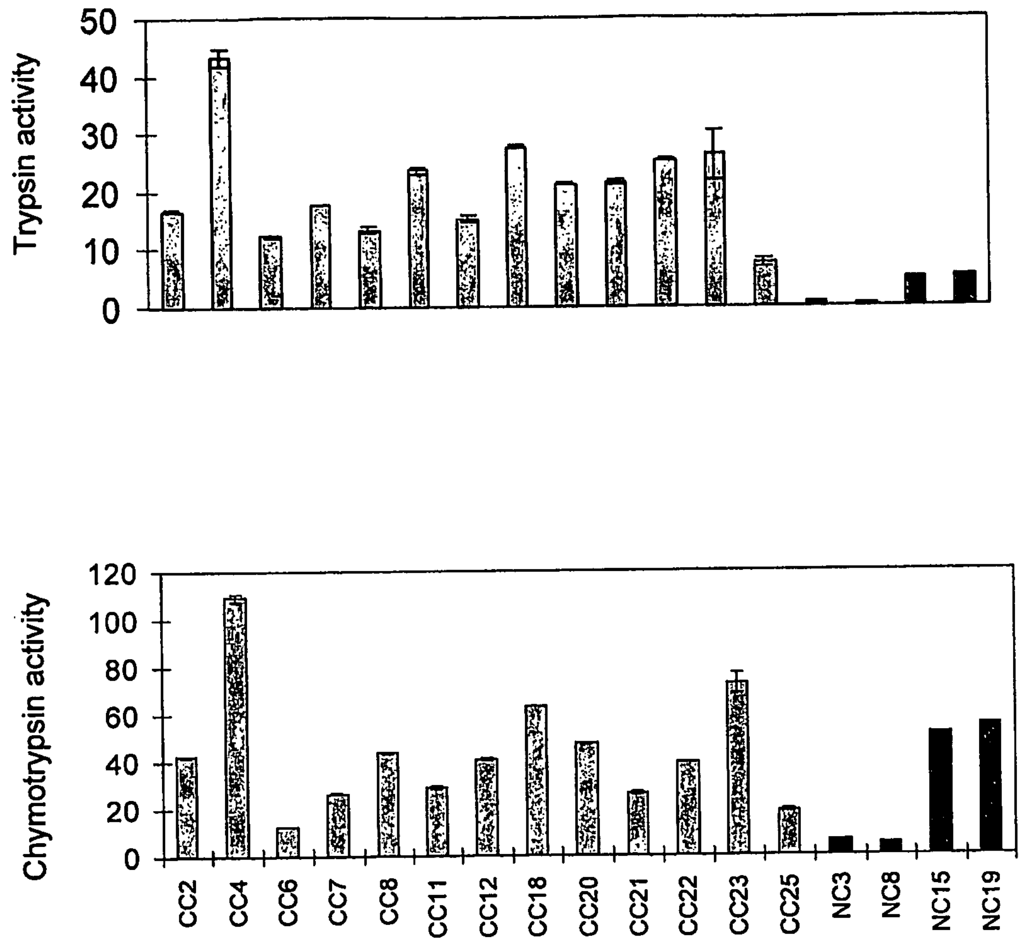


Figure 2B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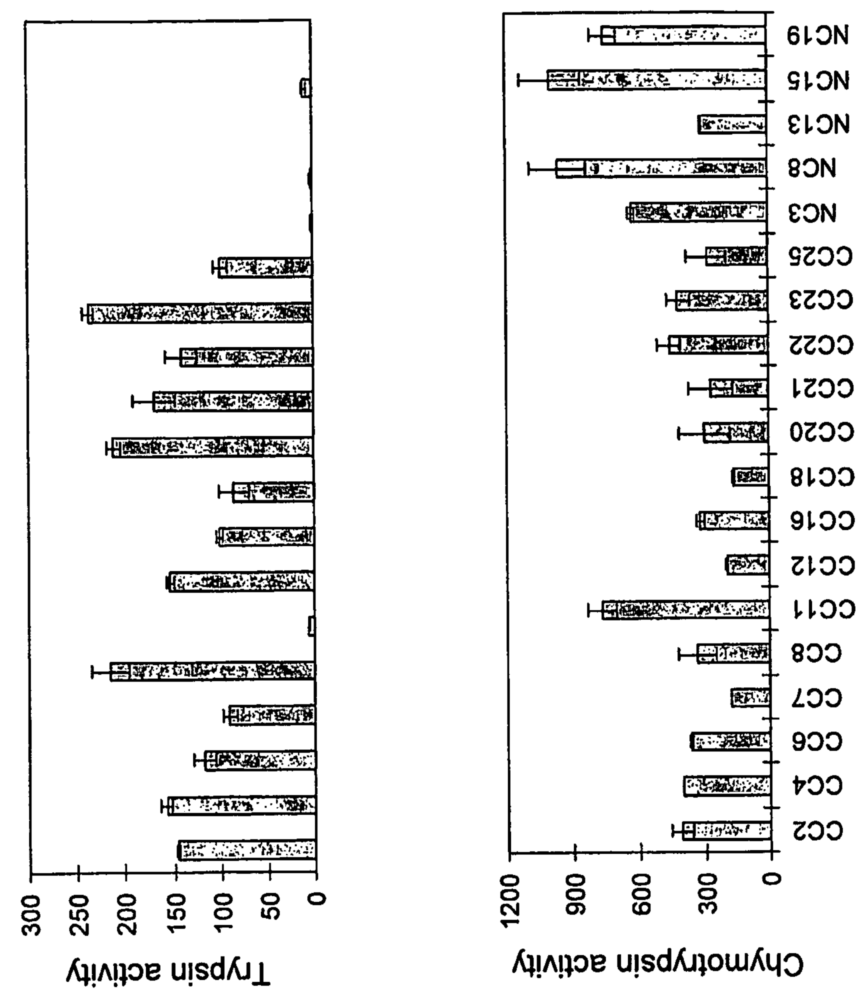
40
30
20
10
0
Chymotrypsin activity
120
100
80
60
40
20
0
CC2
CC4
CC6
CC7
CC8
CC11
CC12
CC18
CC20
CC21
CC22
CC23
CC25
NC3
NC8
NC15
NC19

Figure 2C


Trypsin activity
300
250
200
150
100
50
0
Chymotrypsin activity
1200
900
600
300
0
CC2
CC4
CC6
CC7
CC8
CC11
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CC23
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NC8
NC13
NC15
NC19

Figure 2D

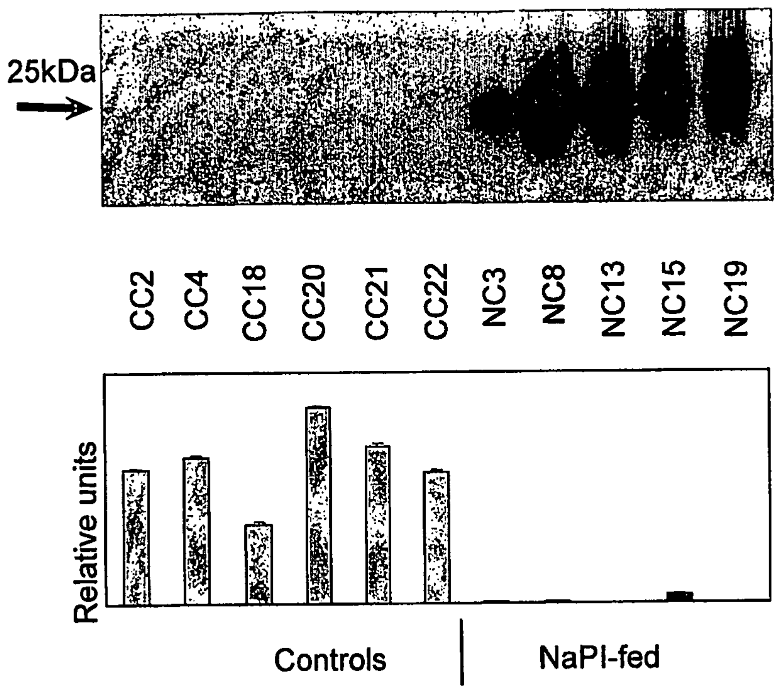
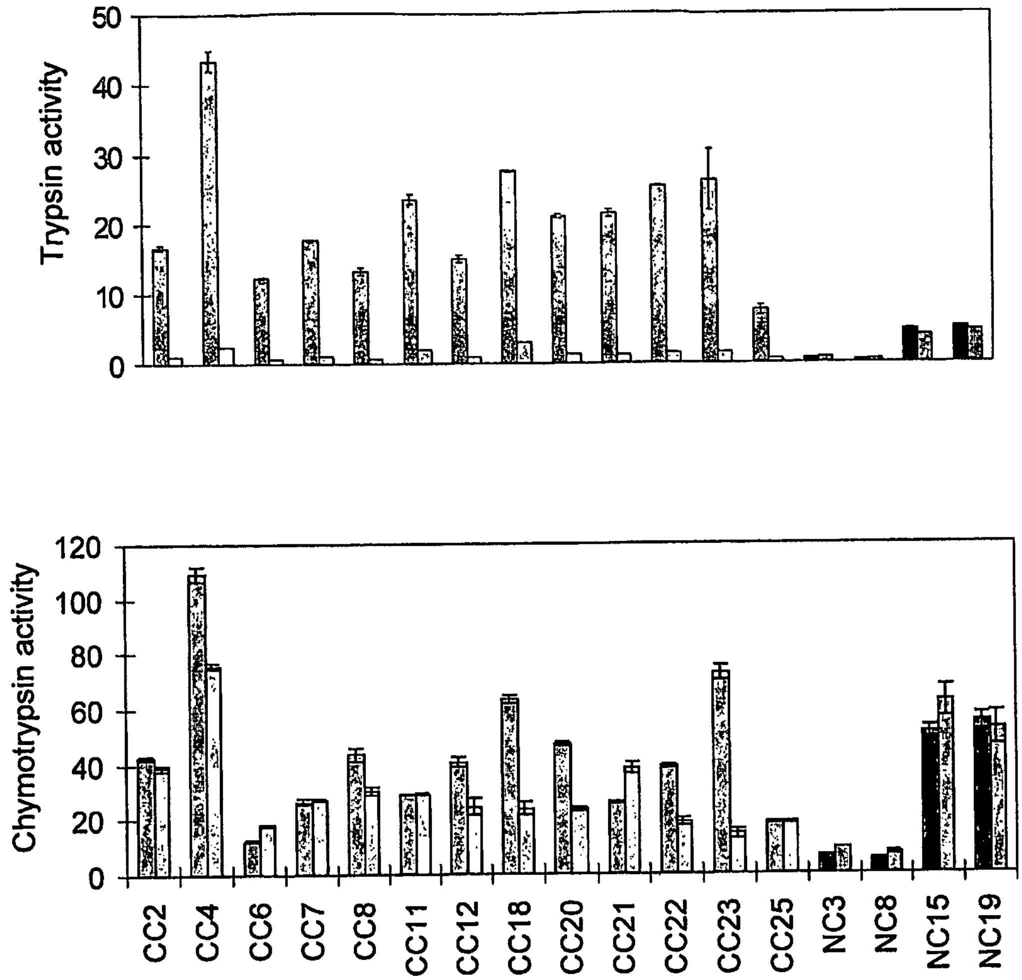

25kDa
CC2
CC4
CC18
CC20
CC21
CC22
NC3
NC8
NC13
NC15
NC19
Relative units
Controls
NaPI-fed

Figure 2E



Trypsin activity
50
40
30
20
10
0
Chymotrypsin activity
120
100
80
60
40
20
0
CC2
CC4
CC6
CC7
CC8
CC11
CC12
CC18
CC20
CC21
CC22
CC23
CC25
NC3
NC8
NC15
NC19

Figure 2F

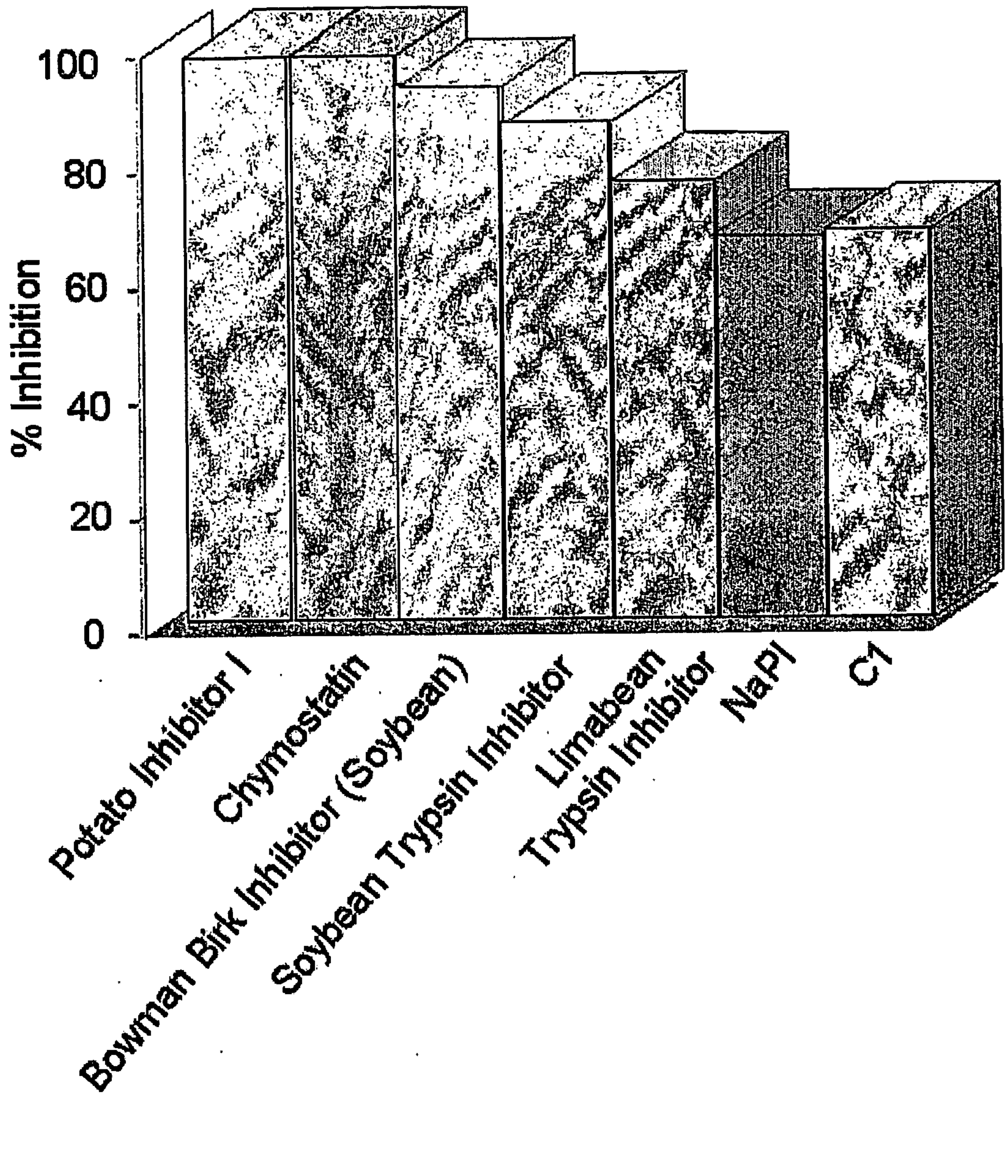

% Inhibition
100
80
60
40
20
0
Potato Inhibitor I
Chymostatin
Bowman Birk Inhibitor (Soybean)
Soybean Trypsin Inhibitor
Limabean Trypsin Inhibitor
NaPI
C1

Figure 3

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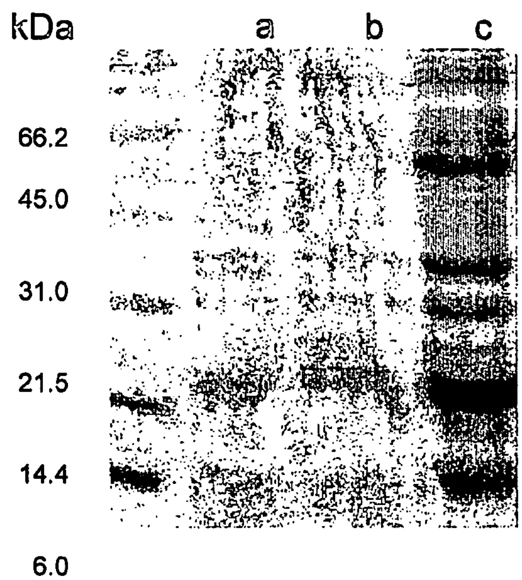


Figure 4A

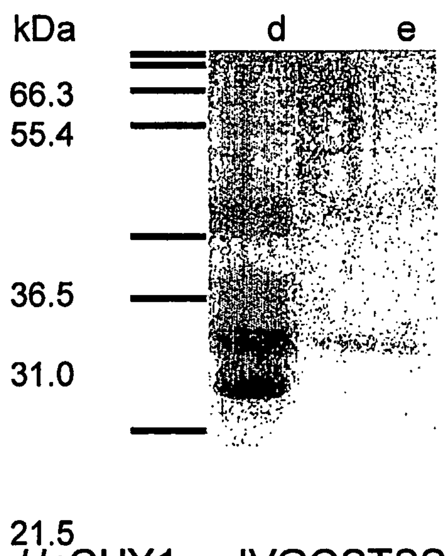
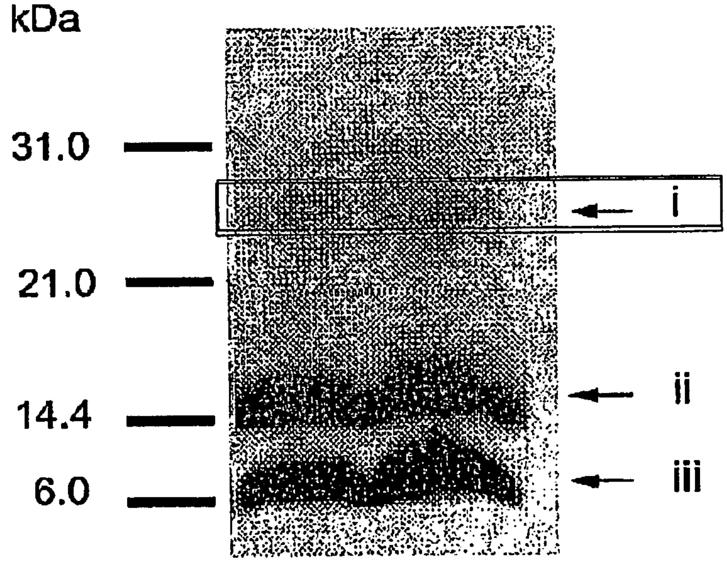


Figure 4B

*Hp*CHY1 IVGGSTSSLGATPYQ

Figure 4C

A



B

i	*Rech1a*	**IVGGSLSSVGQIPYQAGLVIDLAGGQAVCGGSLISA**
	Rech1b	IVGGSISSIGQIPYGAGLVIDFAGGQAVC
	Rech1c	IVGGSTSSVGQFPYQAGLLASFAGGQAVC
	Rech1d	IVGGSVTTLDAYPTIAGLVYNFAGGQAVC

Figure 5

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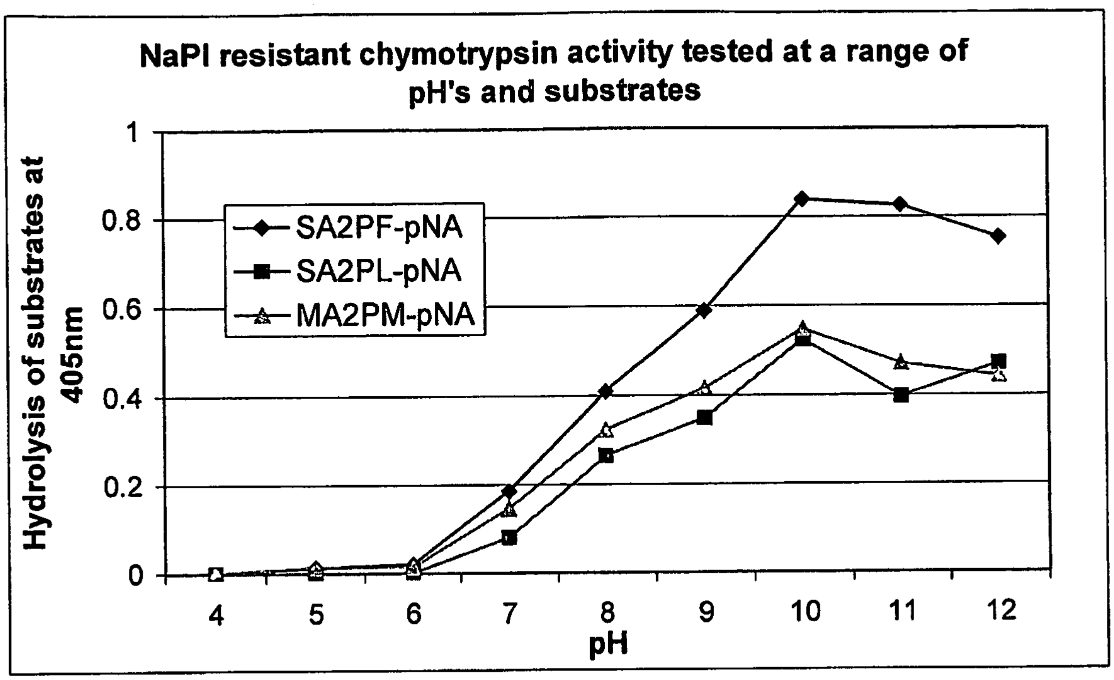

Figure 6

```
CAA72966  MKLLAVTLLAFAAVVSARNIDLEDVIDLEDITAYDYHTKIGIPLAEKIRA
CAA72959  ---LAVTLLAFAAVVSARNIDLEDVIDLEDITAYDYHTKIGIPLAEKIRA
CAA72960  MKLLAVTLLAFAAIVSARNIDLEDVIDLEDITAYDYHTKIGIPLAEKIRA
CAA72958  -----------------INHEAVVDLEDITAYGYHTKVGIPLAEEIRI
CAA72952  MKLFLGVCLTLAVAVSAVEIATPD----ADSPVFGYHAKFGIAEAARIKS
CAA72951  --------------------------------------------------

                            FWY79→  FWG1→
CAA72966  AEEEAERNPSRIVGGSTSSLGAFPYQAGLLATFASGQGVCGGSLLNNRRV
CAA72959  AEEEAERNPSRIVGGSTSSLGAFPYQAGLLATFASGQGVCGGSLLNNRRV
CAA72960  AEEEAERNPSRIVGGSISSLGAFPYQAGLLATFASGQGVCGGSLLNNRRV
CAA72958  AELEASRNPSRIVGGSSASLGQFPYQAGLLINLPLGQSVCGGSLLNQRRV
CAA72952  AEEVQSFNGQRIVGGSITNIANVPYQAGLVITIFIFQSVCGASLISHNRL
CAA72951  ----------------------------------------------HNKWV

CAA72966  LTAAHCWFDGRNQARSFTVVLGSVRLFSGGTRLNTASVVMHGSWNPNLIR
CAA72959  LTAAHCWFDGRNQARSFTVVLGSVRLFSGGTRLNTASVVMHGSWNPNLIR
CAA72960  LTAAHCWFDGRNQARSFTVVLGSVRLFSGGTRLNTASVVMHGSWNPNLIR
CAA72958  LTAAHCWFDGRNQANSLTVILGSINLYFGGTRLNSNSVVMHGSWNPNLIR
CAA72952  VTAAHCKSDGVLTANSFTVVLGSNTLFFGGTRINTNDVVMHPNWNPNTAA
CAA72951  LTAAHCLANRITFVVRFGLTNLTRPEILVESANKYIHPDYDEIRAG-VQT

CAA72966  NDIAMINLPSNVATSGNIAP--IALPSGNELNNNFNGATAVASGFGLARD
CAA72959  NDIAMINLPSNVATSGNIAP--IALPSGNELNNNFNGATAVASGFGLARD
CAA72960  NDIAIINLPSNVATSGNIAP--IALPSGNELNNNFNGATAVASGFGLAND
CAA72958  NDIAIINLPSNVGTSNNIAP--IALPSGNELNNQFAGFTATASGFGRTRD
CAA72952  NDIAVLRISS-VSFSNVIQP--IALPSGDELNNLFVGANALASGFGRTSD
CAA72951  ADLALVGLDHHIEYSANVQPSRLMSSAQKNINYE--GIQMIVSGFGRTDD

                        FWY72→
CAA72966  GGSVDGN---LRHVNLPVITNAVCTVSFP-GIIQSS-NICTSG--ANGRS
CAA72959  GGSVDGN---LRHVNLPVITNAVCTVSFP-GIIQSS-NICTSG--ANGRG
CAA72960  GGSVDGN---LRHVNLPVITNAVCTVSFP-GIIQSS-NICTSG--ANGRS
CAA72958  GGSVSPT---LNHVNLPVITNNVCWQSFP-LYIQSS-NICTSG--ANGRS
CAA72952  SGSIGTN-QQLSSVTIPVITNAQCAAVYGSGFVHAS-NICTSG--AGGKG
CAA72951  LWNGGAASEILLWVYQRGVSNEECLRWYPTSQVIKEETICAGYWDNPSQS

                                                 ←RVG4
CAA72966  TCQGDSGGPLVVTSN-NRRILIGVTSFGSARGCQVGSPAAFARVTSFISW
CAA72959  TCQGDSGGPLVVTSN-NRRILIGVTPFGSARGCQVGSPAAFARVTSFISW
CAA72960  TCQGDSGGPLVVTSN-NRRILIGVTSFGSARGCQVGSPAAFARVTSFISW
CAA72958  TCQGDSGGPLVVTSN-NRRILIGVTSFGSDRGCQVGAPAAFARVTSYISW
CAA72952  TCNGDSGGPLAVDSN-NRKILIGVTSYGAQAGCAAGFPAAFARVTSFVDW
CAA72951  SCQGDSGGPLTIIDADGERTQVGIVSFGSTAGCNSPFPSGYVRPGHYHDW

      ←RVY72
CAA72966  INQRL---------------------------------------------
CAA72959  INQRL---------------------------------------------
CAA72960  INNLL---------------------------------------------
CAA72958  INQRL---------------------------------------------
CAA72952  VQSQ----------------------------------------------
CAA72951  FTEVTGINFDWDSDAIIPGSSESSSSESSEPSSESSESSSEEDGANPSSS

CAA72966  --------------------------------
CAA72959  --------------------------------
CAA72960  --------------------------------
CAA72958  --------------------------------
CAA72952  --------------------------------
CAA72951  EEDGSNPSSEEDAGSPPSEEEEAPEKVRVVEY
```

Figure 7

Primer Name	Sequence 5'-3'
FWG1	TCC CTT ACC AGG C(GCT) GTC [SEQ ID NO:38]
RVG4	TCT GGC GAA GGC AGC AGG [SEQ ID NO:39]
Y79Fw	CTG CTA GCC TCG GAC AAT TC [SEQ ID NO:40]
Y72Fw	CTG GAG TGC AGA CTG CTG AC [SEQ ID NO:41]
Y72Rv	GGA TGA TGG CGT CGC TGT CC [SEQ ID NO:42]

Figure 8

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```
HpF1Apcr        PYQAGLVITIFIFQSVCGASLIPHNRLVTAAHCKSDGVLTANSFTVVLGS 50
HpF1Bpcr        PYQAGLVITIFIFQSVCGASLISHNRLVTAAHCKFDGVMTANSFTVVLGS 50
HpF2Apcr        PYQAGLLANFASGQGVCGGSLLNQRRVLTAAHCWFDGRNQARSFTVVLGS 50
HpF3pcr   ASLGQFPYQAGLLINLPLGQSVCGGSLLNQRRVLTAAHCWFDGRNQATSLTVILGS 57
HpF4pcr         --------------------------------------------------

HpF1Apcr        NTLFFGGTRINTNDVVMHPNWNPSTAANDIAVMRISS-VSFSNVIQPIAL 99
HpF1Bpcr        NTLFFGGTRINTNDVVMHPNWNPSTVANDIAVIRISS-IVFNNVIQPIAL 99
HpF2Apcr        VRLFSGGTRLDTASVVMHGSWNPNLIRNDIAMINLPSNVATSGNIAPIAL 100
HpF3pcr         INLFFGGTRLNSNSVVMHGSWNPNLIRNDIAIINLPSNVGTSGNIAPIAL 100
HpF4pcr         ---------------------SGVQTADLALVGLDQEIEYSANVQPSRL 28

HpF1Apcr        PSGDELNNLFVGANALASGFGRTSDGGSIGSNQQ-VSSVTIPVITNDECA 148
HpF1Bpcr        PSGDELNNLFVGANALASGFGRTSDSGGIGTNQQ-LSSVTIPVITNAECA 148
HpF2Apcr        PSGNELNNNFNGATATASGFGLARDGGSVDGN---LRHVNLPVITNAVCT 147
HpF3pcr         PSGNELNNQFAGFTATASGFGLTRDGGNVSPT---LNHVNLPVITNNVCW 147
HpF4pcr         MSSAQKNINYEGIQMIVSGFGRTDDLWNGGAASEILLWVYQRGVSNEECL 78

HpF1Apcr        AVYGS-AFVHSSNICTSGAGG--KGTCNGDSGGPLAIDSNN-EKILIGVT 194
HpF1Bpcr        AVYGP-AFVHDTNICTSGAGG--KGTCNGDSGGPLAVDSND-KKILIGVT 194
HpF2Apcr        VSFP--GIIQSSNICTSGANG--RSTCQGDSGGP---------------- 177
HpF3pcr         QSFP--LYIQSTNICTSGANG--RGTCQGDSGGPLVVTSNN-RRILIGVT 192
HpF4pcr         RWYPTSQVIKEQTICAGYWDNPSQSSCQGDSGGPLTIIDADGERTQVGIV 128

HpF1Apcr        SYGAQAGCAAGLPAAFARK-----------------------       213
HpF1Bpcr        SYGAADGCAAGFPAASPER-----------------------       213
HpF2Apcr        ------------------------------------------
HpF3pcr         SFGSDRGCQVGAPAAFAR------------------------       210
HpF4pcr         SFGSTAGCNSPFPSGYVRPGHYHDWFTEVTGINFDWDSDAII       120
```

Figure 9

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```
HpCh1AI   ---------- ---AVSA--- -VEIGTPDAD SPVFGYHAKF GIPEAARIKS  33
HpCh1BI   MKLFLGVCLA LAVAVSA--- -VEIGTPEAG SPVFGYHAKF GIAEAARIKS  46
HpCh2A    MKLLAVTLLA FAAVVSARNI DLEDVIDLED ITAYDYHTKI GIPLAEEIRA  50
HpCh2B    MKLLAVTLLA FAAVVSARNI DLEDVIDLED ITAYDYHTKI GIPLAEKIRA  50
HpCh3A    ---------- ---------- ---------- ---------- ----------
HpCh4I    MAAAYLLGLL FVLGYVQGGL LNADPAIIED LRDA------ ----------  34
HpCh4II   MAAAYLLGLL FVLGYVQGGL LNADPAIIED LRDA------ ----------  34

                     ↓↓                                ⊙
HpCh1AI   AEEVQSFNGQ RIVGGSITDI ANVPYQAGLV ITIFIF-QSV CGASLISHNR  82
HpCh1BI   AEEVQSFNGQ RIVGGSITNI ANVPYQAGLV ITIFIF-QSV CGASLISHNR  95
HpCh2A    AEEEAERDPS RIVGGSTSSL GAFPYQAGLL ANFASG-QGV CGGSLLNQRR  99
HpCh2B    AEEEAERNPS RIVGGSTSSL GAFPYQAGLL ASFASG-QGV CGGSLLNVRR  99
HpCh3A    ---------- -IVGGSSASL GQFPYQAGLL INLPLG-QSV CGGSLLNQRR  38
HpCh4I    --EFSSG--S RIVAGWPAVE GQIPYQGSLR MVSAIGGVSS CGCSLIHNKW  80
HpCh4II   --EFSSF--S RIVAGWPAVE GQIPYQGSLR MVSAIGGVSS CGCSLIHNKW  80

                 ▼●
HpCh1AI   LVTAAHCKSD GVLTANSFTV VLGSNTLFFG GTRINTNDVV MHPNWNPS--  130
HpCh1BI   LVTAAHCKFD GVMTANSFTV VLGSNTLFFG GTRINTNDVV MHPNWNPS--  143
HpCh2A    VLTAAHCWFD GRNQARSFTV VLGSVRLFSG GTRLDTASVV MHGSWNPN--  147
HpCh2B    VLTAAHCWFD GRNQARSFTV VLGSVRLYSG GTRLNTASVV MHGSWNPN--  147
HpCh3A    VLTAAHCWFD GRNQATSLTV ILGSINLFFG GTRLNSNSVV MQGSWNPN--  86
HpCh4I    VLTAAHCLAN ----RITFVV RFGLTNLTRP EILVESTNKY IHPEYDEIRA  126
HpCh4II   VLTAAHCLAN ----RITFVV RFGLTNLTRP EILVESTNKY IHPEYDEIRA  126

              ▼
HpCh1AI   -TAANDIAVM RISS-VSFSN VIQPIALPSG DELNNLFVGA NALASGFGRT  178
HpCh1BI   -TVANDIAVI RISS-IVYNN VIQPIALPSG DELDNLFVGA NALASGFGRT  191
HpCh2A    -LIRNDIAMI NLPSNVATSG NIAPIALPSG NELNNNFNGA TATASGFGLA  196
HpCh2B    -LVRNDIAMI NLPSNVATSG NIAPIALPSG NELNNQFAGA TATASGFGLA  196
HpCh3A    -LIRNDIAII NLPSNVGTSG NIAPIALPSG NELNNQFAGF TATASGFGLT  135
HpCh4I    GVQTADLALV GLDHEIEYSA NVQPSRLMSS AQKNINYEGI QMIVSGFGRT  176
HpCh4II   GVQTADLALV GLDQEIEYSA NVQPSRLMSS AQKNINYEGI QMIVSGFGRT  176

                                 ●                      ●
HpCh1AI   SDGGSIGSNQ Q-VSSVTIPV ITNDECAAVY GS-AFVHSSN ICTSGAGG--  224
HpCh1BI   SDSGGIGTNQ Q-LSSVTIPV ITNAECAAVY GP-AFVHDTN ICTSGAGG--  237
HpCh2A    RDGGSVDGN- --LRHVNLPV ITNAVCTVSF P--GIIQSSN ICTSGANG--  239
HpCh2B    RDGGVIDGN- --LRHVNLPV ITNAVCSQSF P--GLIQASN VCTSGANG--  239
HpCh3A    RDGGNVSPT- --LNHVNLPV ITNNVCWQSF P--LYIQSTN ICTSGANG--  178
HpCh4I    DDLWNGGAAS EILLWVYQRG VSNEECLRWY PTSQVIKEQT ICAGYWDNPS  226
HpCh4II   DDLWNGGAAS EILLWVYQRG VSNEECLRWY PTSQVIKEQT ICAGYWDNPS  226

            ¥ ●   ▼                       §       ●           #
HpCh1AI   KGTCNGDSGG PLAVDSNNEK IL-IGVTSYG AQAGCAVG-- ---LPAAFAR  268
HpCh1BI   KGTCNGDSGG PLAVDSNDKK IL-IGVTSYG AADGCAAG-- ---FPAAFAR  281
HpCh2A    RSTCQGDSGG PLVVNSNNRR IL-IGVTSFG SARGCQVG-- ---SPAAFAR  283
HpCh2B    RSTCQGDSGG PLVVNSNNRR IL-IGVTSFG SARGCQVG-- ---SPAAFAR  283
HpCh3A    RGTCQGDSGG PLVVTSNNRR IL-IGVTSFG SDRGCQVG-- ---APAAFAR  222
HpCh4I    QSSCQGDSGG PLTIIDADGE RTQSRYCELR --IHCWNA-- -AHSPQGYVR  272
HpCh4II   QSSCQGDSGG PLTIIDADGE RTQVGIVSSD PLLDATVHSP RVTSPGHYHD  276

HpCh1AI   VTSFVSWVQS Q--------- ---------- ---------- ----------  279
HpCh1BI   VTSFVSWVQS Q--------- ---------- ---------- ----------  292
HpCh2A    VTSFISWINQ RL-------- ---------- ---------- ----------  295
HpCh2B    VSSYISWINQ RL-------- ---------- ---------- ----------  295
HpCh3A    VTSYISWINQ RL-------- ---------- ---------- ----------  234
HpCh4I    PGHYHDWFTE VTGINFDWDS DAIIP----- ---------- ----------  297
HpCh4II   GHRGDRHQLR LGQRRHYPDS SESSLRAAIL PLESSRAFIR RNQSSFRGGL  326
```

Figure 10A

```
HpCh1AI   ---------- ---------- ---------- --------- 279
HpCh1BI   ---------- ---------- ---------- --------- 292
HpCh2A    ---------- ---------- ---------- --------- 295
HpCh2B    ---------- ---------- ---------- --------- 295
HpCh3A    ---------- ---------- ---------- --------- 234
HpCh4I    ---------- ---------- ---------- --------- 297
HpCh4II   CQPPRFPTRT VPTHLPRRTL AAPPSEEEEA PEKVRVVEY 365
```

Figure 10B

		F1 F2
[SEQ ID NO:55]	*Rech1a*	IVGGSLSSVG QIPYQAGLVI DLAGGQAV~C GGSLISA
[SEQ ID NO:56]	*Rech1b*	IVGGSTSSVG QFPYQAGLLA SFAGGQAV~C G
[SEQ ID NO:57]	*Family1a*	IVGGSITDIA NVPYQAGLVI TIFIFQSV~C GASLISHN
[SEQ ID NO:58]	*Family1b*	IVGGSITNIA NVPYQAGLVI TIFIFQSV~C GASLISHN
[SEQ ID NO:59]	*Family2a*	IVGGSTSSLG AFPYQAGLLA SFASGQGV~C GGSLLNVR
[SEQ ID NO:60]	*Family2b*	IVGGSTSSLG AFPYQAGLLA NFASGQGV~C GGSLLNQR
[SEQ ID NO:61]	*Family3*	IVGGSSASLG QFPYQAGLSL IY~SGQSV~C GGSLLNQRR
[SEQ ID NO:62]	*Family4*	IVAGWPAVEG QIPYQGSLRM VSAIGGVSSC GCSLIHN

Figure 11A

N-terminal sequence of resistant chymotrypsin (Rech 1a) F1 F2 IVGGSLSSVGQIPYQAGLVIDLAGGQAVCGGSLISA [SEQ ID NO:9]	
Primer Name	**Oligonucleotide sequence 5'-3'**
Fw2ResChy = F1	TC(AGCT) GT(AGCT) GG(AGCT) CA(AG) AT(ACT) CC [SEQ ID NO:10]
FwResChym = F2	GT(AGCT) AT(ACT) GA(CT) CT(AGCT) GC(AGCT) GG(AGCT) GG [SEQ ID NO:11]

Figure 11B

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```
HpCHF5  GTTCACCTCGAGGATTCTATTGATCTGGAAGATATTACCGCTTGGGGATA 50
         V  H  L  E  D  S  I  D  L  E  D  I  T  A  W  G  Y
   -40                                              -24
HpCHF5  CCTCACCAAATTCGGTATTCCAGAAGCTGAGAAAATCCGCAACGCTGAAG 100
         L  T  K  F  G  I  P  E  A  E  K  I  R  N  A  E
                                                   -8
HpCHF5  AAGCTAGCTCTGCTAGCAGGATCGTCGGTGGTTCATTGTCCAGTGTCGGA 150
        E  A  S  S  A  S  R  I  V  G  G  S  L  S  S  V  G
                        -1 | +1                    +10
HpCHF5  CAGATCCCTTACCAGGCTGGTCTCGTCATTGACTTAGCAGGTGGCCAGGC 200
        Q  I  P  Y  Q  A  G  L  V  I  D  L  A  G  G  Q  A

HpCHF5  TGTCTGCGGAGGCTCCCTGATCAGCGCTTCCCGCGTACTGACCGCTGCTC 250
         V  C  G  G  S  L  I  S  A  S  R  V  L  T  A  A

HpCHF5  ACTGCTGGTTCGACGGCCAAAACCAGGCCTGGAGATTCACCGTTGTTCTT 300
        H  C  W  F  D  G  Q  N  Q  A  W  R  F  T  V  V  L
        #
HpCHF5  GGTTCCACCACCTTGTTCTCTGGCGGTACCAGAATCCCTACATCCAATGT 350
         G  S  T  T  L  F  S  G  G  T  R  I  P  T  S  N  V

HpCHF5  TGTTATGCACGGAAGCTGGACTCCTAGCCTTATCCGTAACGATGTTGCCG 400
          V  M  H  G  S  W  T  P  S  L  I  R  N  D  V  A
                                                 #
HpCHF5  TAATCAGATTGGGCACCAACGTAGCAACCTCAAACACCATTGCCATCATC 450
        V  I  R  L  G  T  N  V  A  T  S  N  T  I  A  I  I

HpCHF5  GCTCTACCCAGCGGCAGCCAGATCAACGAGAACTTCGCCGGTGAAACCGC 500
         A  L  P  S  G  S  Q  I  N  E  N  F  A  G  E  T  A

HpCHF5  CCTCGCCTCCGGCTTCGGTCTCACCAGTGACACCGGCAGCATCTCCAGCA 550
          L  A  S  G  F  G  L  T  S  D  T  G  S  I  S  S

HpCHF5  ACCAGGCTCTGAGCCACGTCAACCTGCCAGTGATCACCAACGCTGTGTGC 600
        N  Q  A  L  S  H  V  N  L  P  V  I  T  N  A  V  C

HpCHF5  AGAAATTCATTCCCCCTGCTGATCCAGGACTCTAACATTTGCACCAGCGG 650
         R  N  S  F  P  L  L  I  Q  D  S  N  I  C  T  S  G

HpCHF5  TGCCAACGGCAGGAGCACTTGCCGCGGTGACTCCGGCGGTCCTCTCGTCG 700
          A  N  G  R  S  T  C  R  G  D  S  G  G  P  L  V
                        §                 #
HpCHF5  TCACCAGGAACAACAGACCACTCTTGATCGGTATCACCTCTTTCGGATCT 750
        V  T  R  N  N  R  P  L  L  I  G  I  T  S  F  G  S

HpCHF5  GCCCGCGGTTGCCAAGTTGGATCTCCCGCTGCCTTCGCCAGAGTCACCTC 800
         A  R  G  C  Q  V  G  S  P  A  A  F  A  R  V  T  S

HpCHF5  TTACATCAGCTGGATCAACGGCCAGCTCTAAAATATCGAACATTTTGCCA 850
          Y  I  S  W  I  N  G  Q  L  *

HpCHF5  TATCTACAGAGATATTTTGAAATACGTTAATTTAAATAAATATTTTATTT 900
HpCHF5  ATTCAAAAAAAAAAAAAAAAA                              921
```

Figure 12

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```
HpCh1AI      IVGGSITDIANVPYQAGLVITIFI-FQSVCGASLISHNRLVTAAHCKSDG  49
HpCh1BI      IVGGSITNIANVPYQAGLVITIFI-FQSVCGASLISHNRLVTAAHCKFDG  49
HpCh2A       IVGGSTSSLGAFPYQAGLLANFAS-GQGVCGGSLLNQRRVLTAAHCWFDG  49
HpCh2B       IVGGSTSSLGAFPYQAGLLASFAS-GQGVCGGSLLNVRRVLTAAHCWFDG  49
HpCh3        IVGGSSASLGQFPYQAGLSLIYS--GQSVCGGSLLNQRRVLTAAHCWFDG  48
HpCh4I       IVAGWPAVEGQIPYQGSLRMVSAIGGVSSCGCSLIHNKWVLTAAHCLAN-  49
HpCh5        IVGGSLSSVGQIPYQAGLVIDLAG-GQAVCGGSLISASRVLTAAHCWFDG  49
TrypsinIV    IVGGYTCEENSLPYQVSLNSGSH-----FCGGSLISEQWVVSAAHCY---  42

HpCh1AI      VLTANSFTVVLGSNTLFFGG-TRINTNDVVMHPNWNPS---TAANDIAVM  95
HpCh1BI      VMTANSFTVVLGSNTLFFGG-TRINTNDVVMHPNWNPS---TVANDIAVI  95
HpCh2A       RNQARSFTVVLGSVRLFSGG-TRLDTASVVMHGSWNPN---LIRNDIAMI  95
HpCh2B       RNQARSFTVVLGSVRLYSGG-TRLNTASVVMHGSWNPN---LVRNDIAMI  95
HpCh3        RNQATSLTVILGSINLFFGG-TRLNSNSVVMHGSWNPN---LIRNDIAII  94
HpCh4I       ---RITFVVRFGLTNLTRPE-ILVESTNKYIHPEYDEIRAGVQTADLALV  95
HpCh5        QNQAWRFTVVLGSTTLFSGG-TRIPTSNVVMHGSWTPS---LIRNDVAVI  95
TrypsinIV    -KTRIQVRLGEHNIKVLEGNEQFINAAKIIRHPKYNRD---TLDNDIMLI  88

HpCh1AI      RISS-VSFSNVIQPIALPSGDELNNLFVGANALAFGFGRTSDGGSIGSNQ  144
HpCh1BI      RISS-IVYNNVIQPIALPSGDELDNLFVGANALASGFGRTSDSGGIGTNQ  144
HpCh2A       NLPSNVATSGNIAPIALPSGNELNNNFNGATATASGFGLARDGGSVDGN-  144
HpCh2B       NLPSNVATSGNIAPIALPSGNELNNQFAGATATASGFGLARDGGVIDGN-  144
HpCh3        NLPSNVGTSGNIAPIALPSGNELNNQFAGFTATASGFGLTRDGGNVSPT-  143
HpCh4I       GLDHEIEYSANVQPSRLMSSAQKNINYEGIQMIVSGFGRTDDLWNGGAAS  145
HpCh5        RLGTNVATSNTIAIIALPSGSQINENFAGETALASGFGLTSDTGSISSNQ  145
TrypsinIV    KLSSPAVINARVSTISLPTAP----PAAGTECLISGWGNTLSFGADYPD-  133

HpCh1AI      Q-VSSVTIPVITNDECAAVYG-SAFVHSSNICT--SGAGGKGTCNGDSGG  190
HpCh1BI      Q-LSSVTIPVITNAECAAVYG-PAFVHDTNICT--SGAGGKGTCNGDSGG  190
HpCh2A       --LRHVNLPVITNAVCTVSF--PGIIQSSNICT--SGANGRSTCQGDSGG  188
HpCh2B       --LRHVNLPVITNAVCSQSF--PGLIQASNVCT--SGANGRSTCQGDSGG  188
HpCh3        --LNHVNLPVITNNVCWQSF--PLYIQSTNICT--SGANGRGTCQGDSGG  187
HpCh4AI      EILLWVYQRGVSNEECLRWYPTSQVIKEQTICAGYWDNPSQSSCQGDSGG  195
HpCh5        A-LSHVNLPVITNAVCRNSF--PLLIQDSNICT--SGANGRSTCRGDSGG  190
TrypsinIV    E-LKCLDAPVLTQAECKASY--PGKITNSMFCVG-FLEGGKDSCQRDSGG  179
                                                      ↑↑
HpCh1AI      PLAIDSNN--EKILIGVTSYGAQAGCAAGLPAAFARVTSFVSWVQSQ---  235
HpCh1BI      PLAVDSND--KKILIGVTSYGAADGCAAGFPAAFARVTSFVSWVQSQ---  235
HpCh2A       PLVVNSNN--RRILIGVTSFGSARGCQVGSPAAFARVTSFISWINQRL--  234
HpCh2B       PLVVNSNN--RRILIGVTSFGSARGCQVGSPAAFARVSSYISWINQRL--  234
HpCh3        PLVVTSNN--RRILIGVTSFGSDRGCQVGAPAAFARVTSYISWINQRL--  233
HpCh4I       PLTIIDADGERTQSRYCELRIHCWNATAHSPQGYVRPGHYHDWFTEVTGI  245
HpCh5        PLVVTRNN--RPLLIGITSFGSARGCQVGSPAAFARVTSYISWINGQL--  236
TrypsinIV    PVVCNG------QLQGVVSWGHGCAWKN-RPGVYTKVYNYVDWIKDTIAA  222

HpCh1AI      -----------  235
HpCh1BI      -----------  235
HpCh2A       -----------  234
HpCh2B       -----------  234
HpCh3        -----------  233
HpCh4I       NFDWDSDAIIP  256
HpCh5        -----------  236
TrypsinIV    NS---------  224
```

Figure 13

10/554237


```
[SEQ ID NO:71]  DH04_H02 ARMIGERA  VHLEDSIDLE DITAWGYLTK FGIPEAEKIR NAEEASSASR
[SEQ ID NO:72]  RECH1A PUNCTIGERA  .......... .......... .......... ..........

                DH04_H02 ARMIGERA  IVGGSLSSLG QIPYQAGLVI DLSGGQAVCG GSLISASRVL
                RECH1A PUNCTIGERA  .......... .......... ..A....... ..........

                DH04_H02 ARMIGERA  TAAHCWFDGQ NQAWRFTVVL GSTTLFSGGT RIATSNVVMH
                RECH1A PUNCTIGERA  .......... .......... .......... ..P.......

                DH04_H02 ARMIGERA  GSWTPSLIRN DVAVIRLGTN VGTSNTIAII ALPSGSQINE
                RECH1A PUNCTIGERA  .......... .......... .......... ..........

                DH04_H02 ARMIGERA  NFAGETALAS GFGLTSDSGS ISSNQALSHV NLPVITNAVC
                RECH1A PUNCTIGERA  .......... .......T.. .......... ..........

                DH04_H02 ARMIGERA  RSSFPLLIQD SNICTSGANG RSTCRGDSGG PLVVTRNSRP
                RECH1A PUNCTIGERA  .N........ .......... .......... .......N..

                DH04_H02 ARMIGERA  LLIGITSFGS ARGCQVGSPA AFARVTSYIS WINGQ
                RECH1A PUNCTIGERA  .......... .......... .......... .....
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Figure 14

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21/36

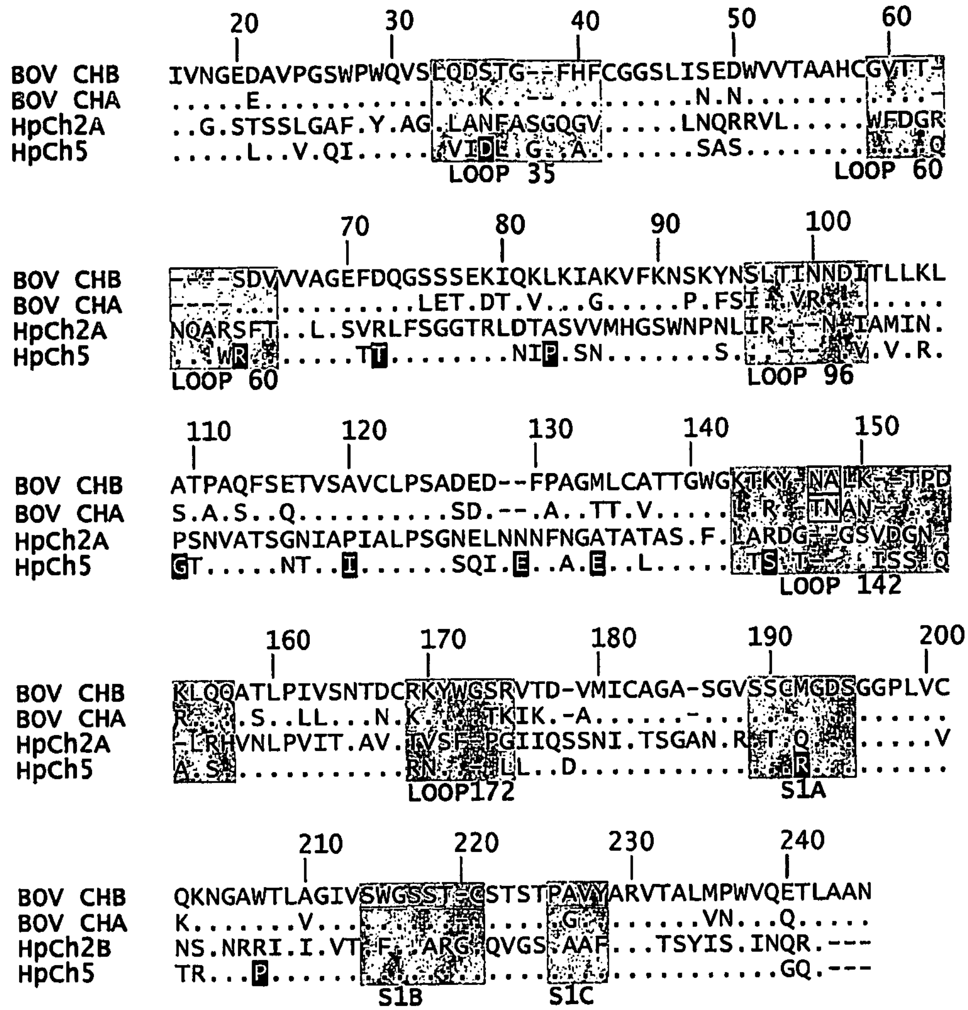

Figure 15

10/554237

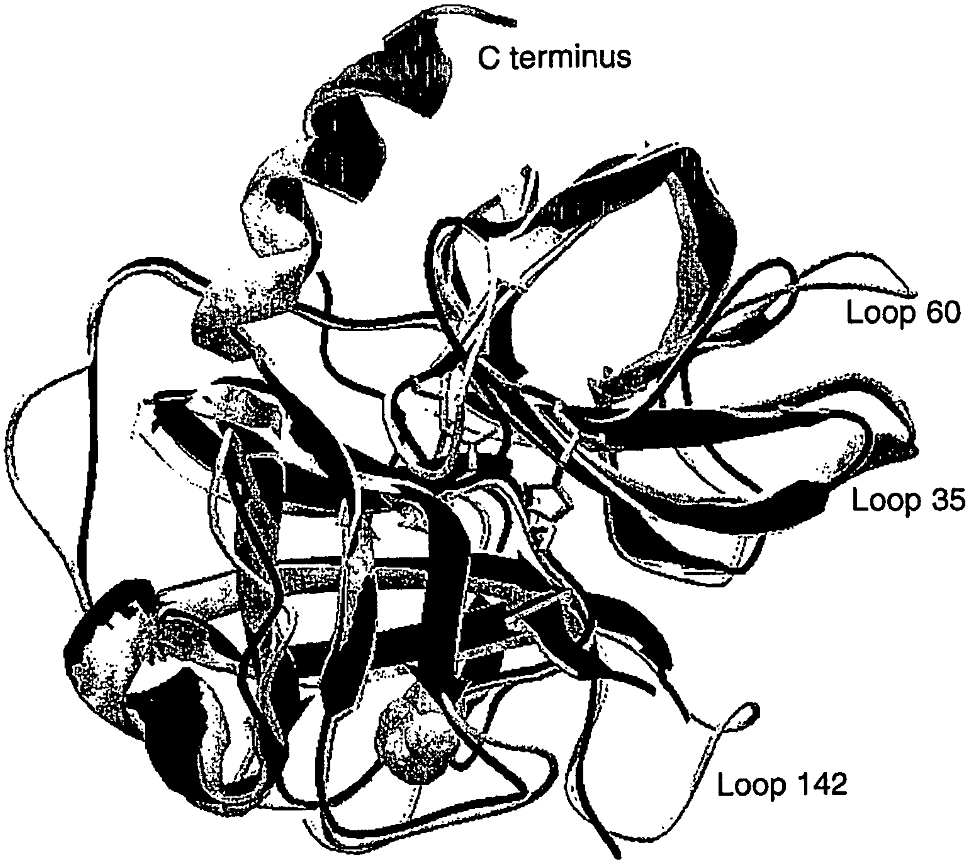

Figure 16

Figure 17

10/554237

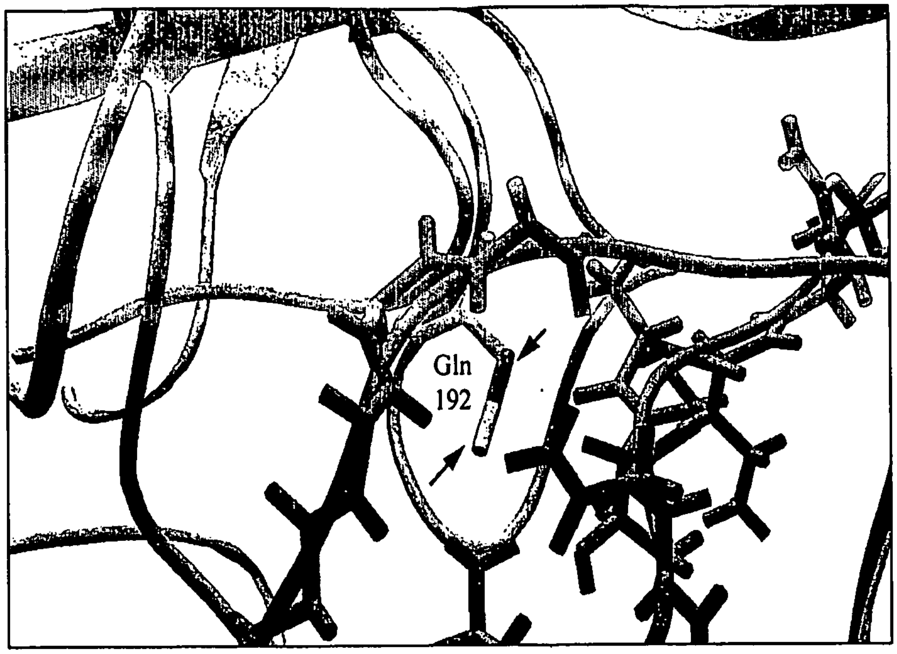

Figure 18A

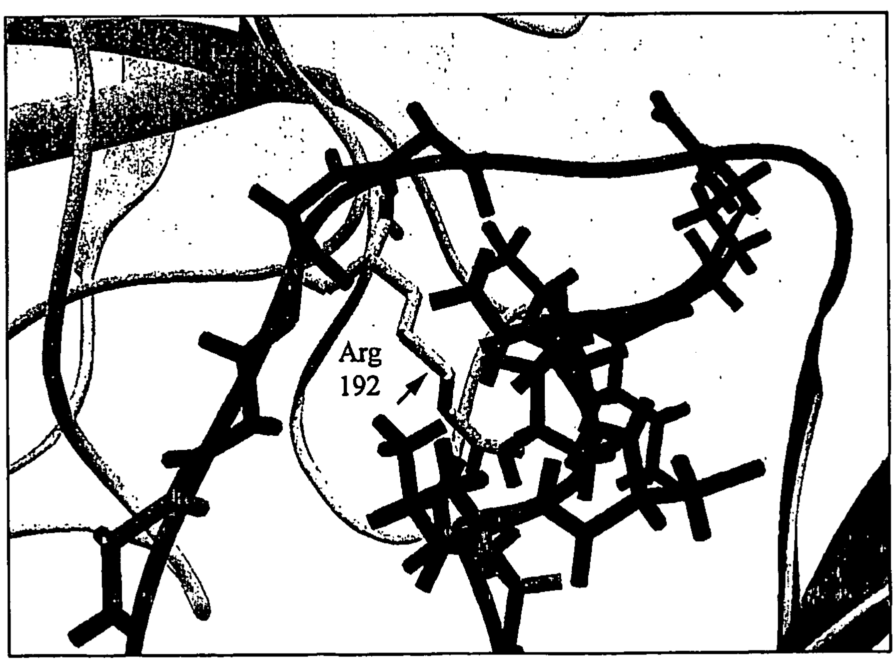

Figure 18B

10/554237

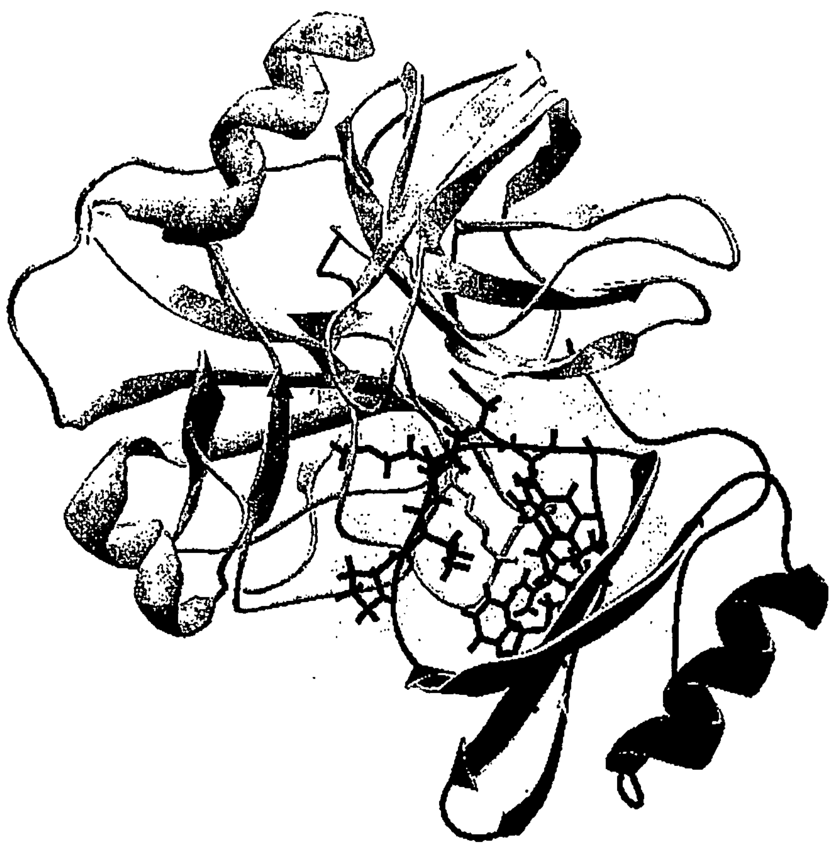

Figure 19A

10/554237

Figure 19B

10/554237

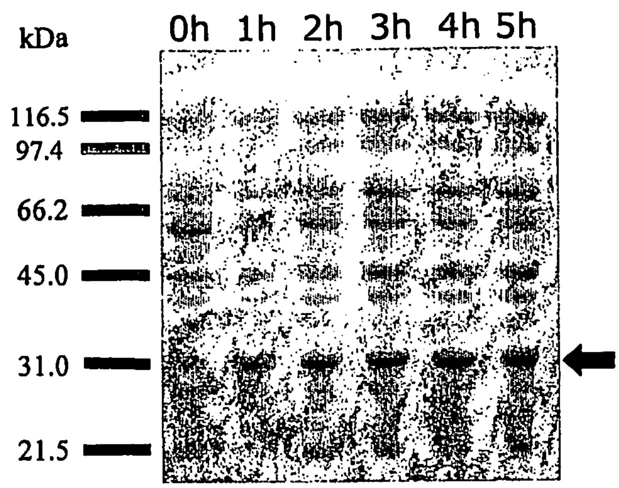

Figure 20A

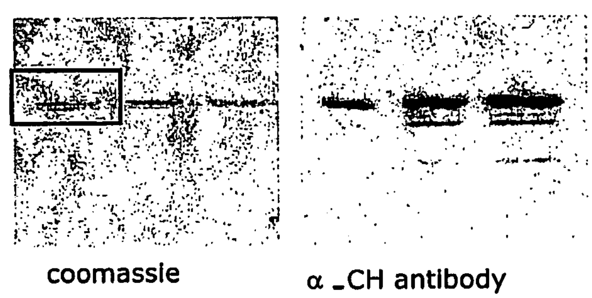

Figure 20B

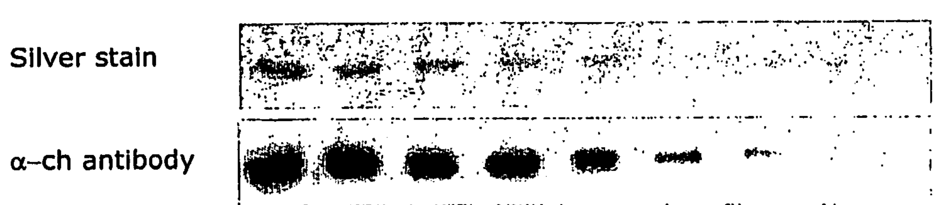

Figure 20C

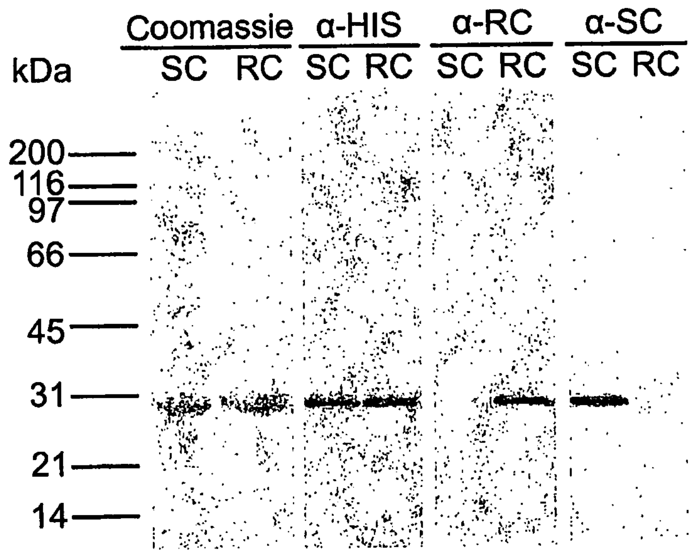
Coomassie
α-HIS
α-RC
α-SC
kDa
SC RC SC RC SC RC SC RC
200
116
97
66
45
31
21
14

Figure 21

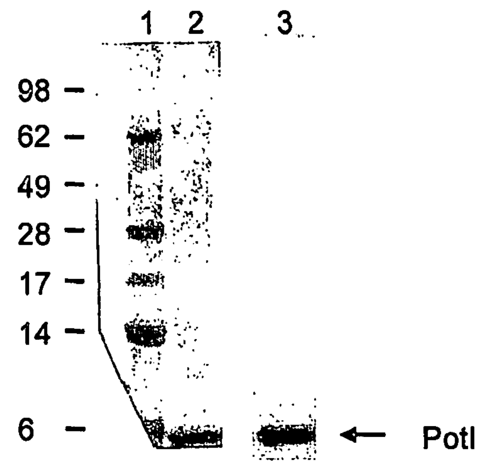

Figure 22A

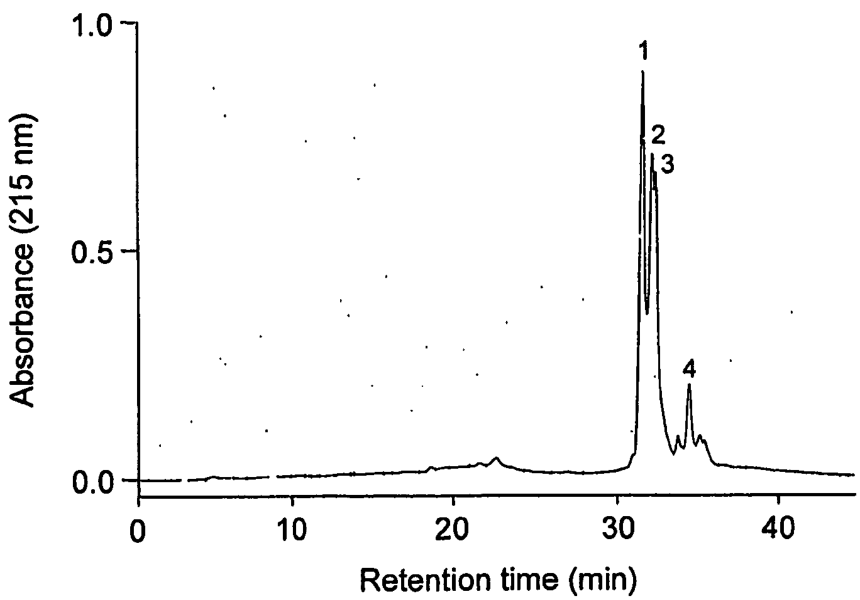

Figure 22B

10/554237

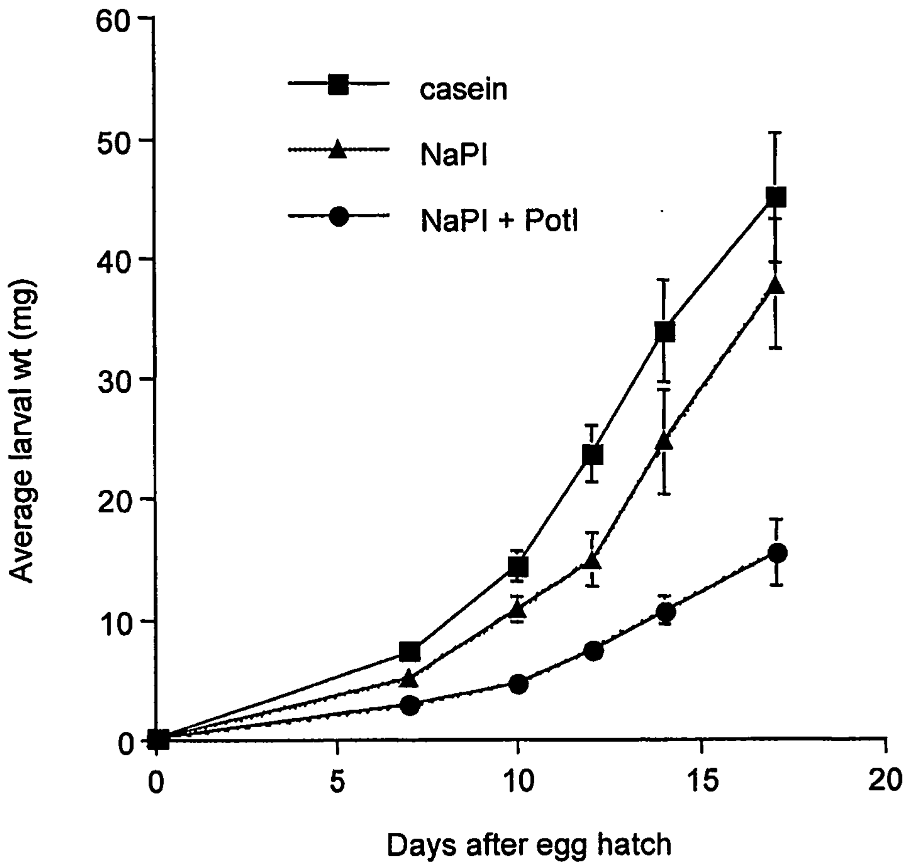

Figure 23

10/554237

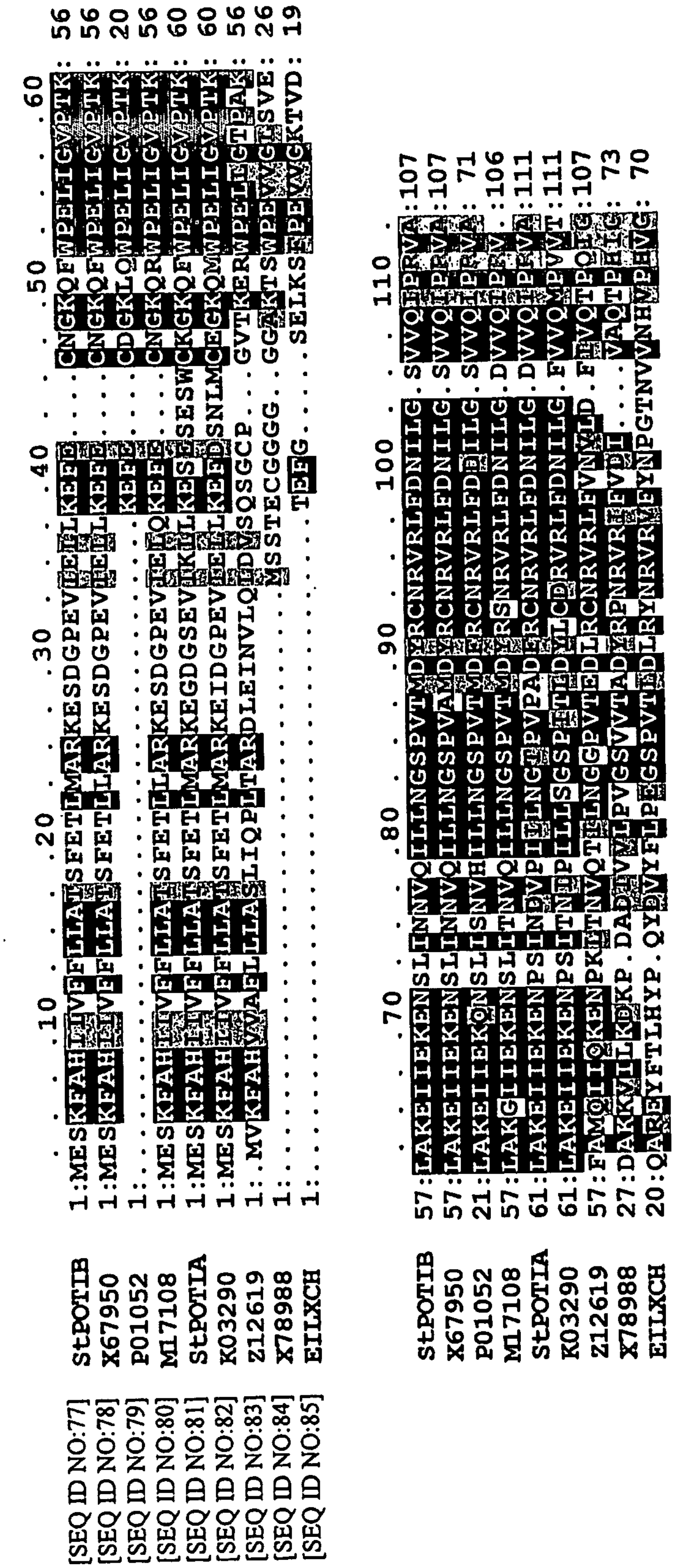

Figure 24

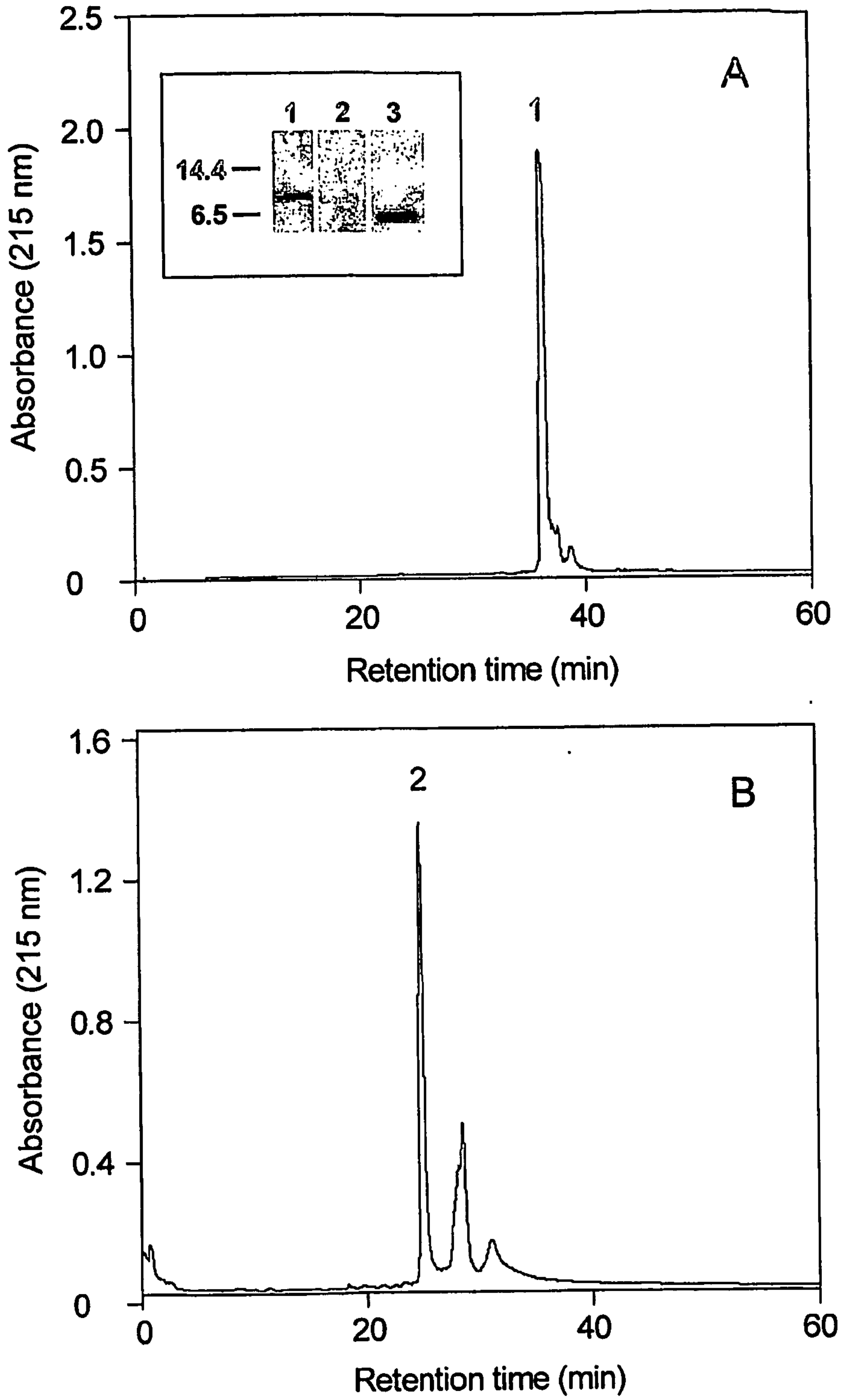
A
1
1 2 3
14.4
6.5
Absorbance (215 nm)
2.5
2.0
1.5
1.0
0.5
0
0
20
40
60
Retention time (min)
B
2
Absorbance (215 nm)
1.6
1.2
0.8
0.4
0
0
20
40
60
Retention time (min)

Figure 25

10/554237

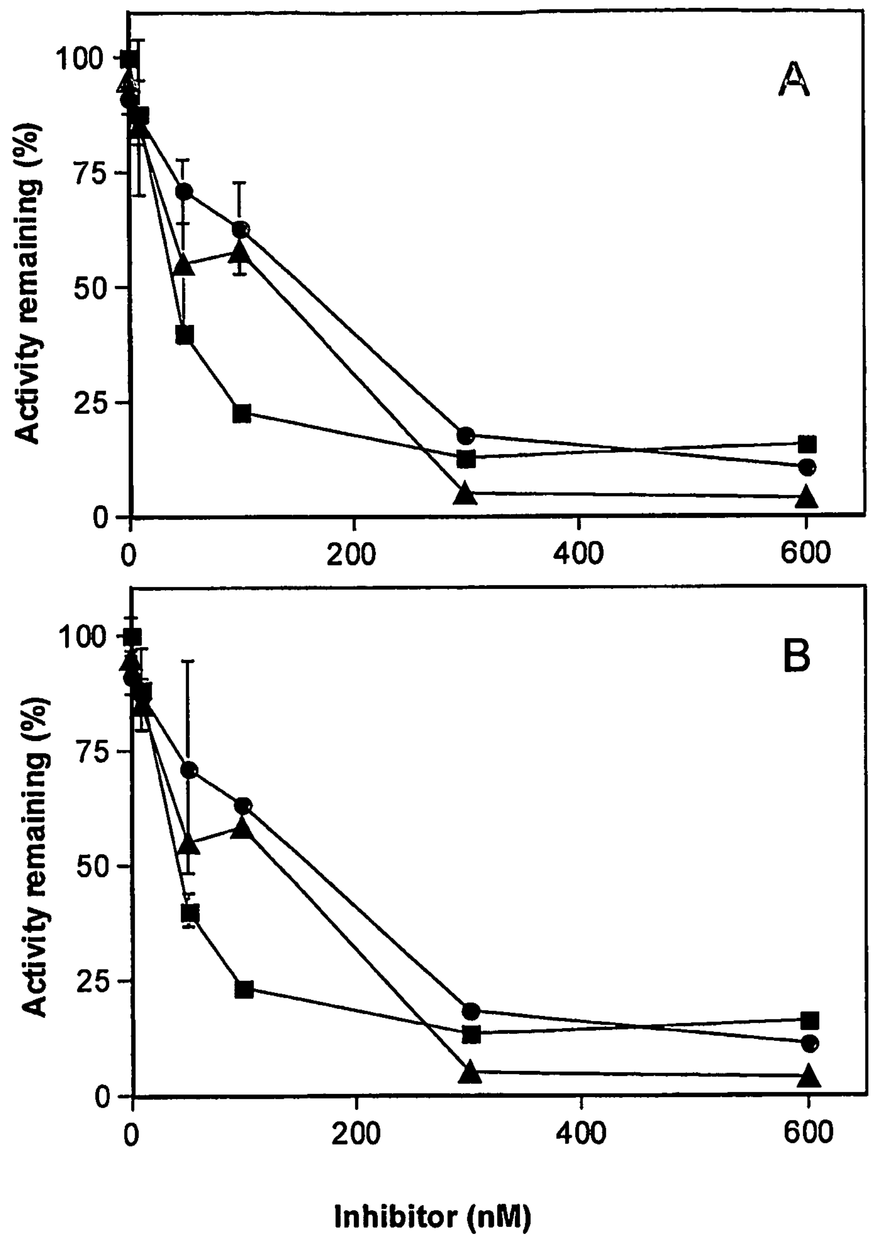

Figure 26

10/554237

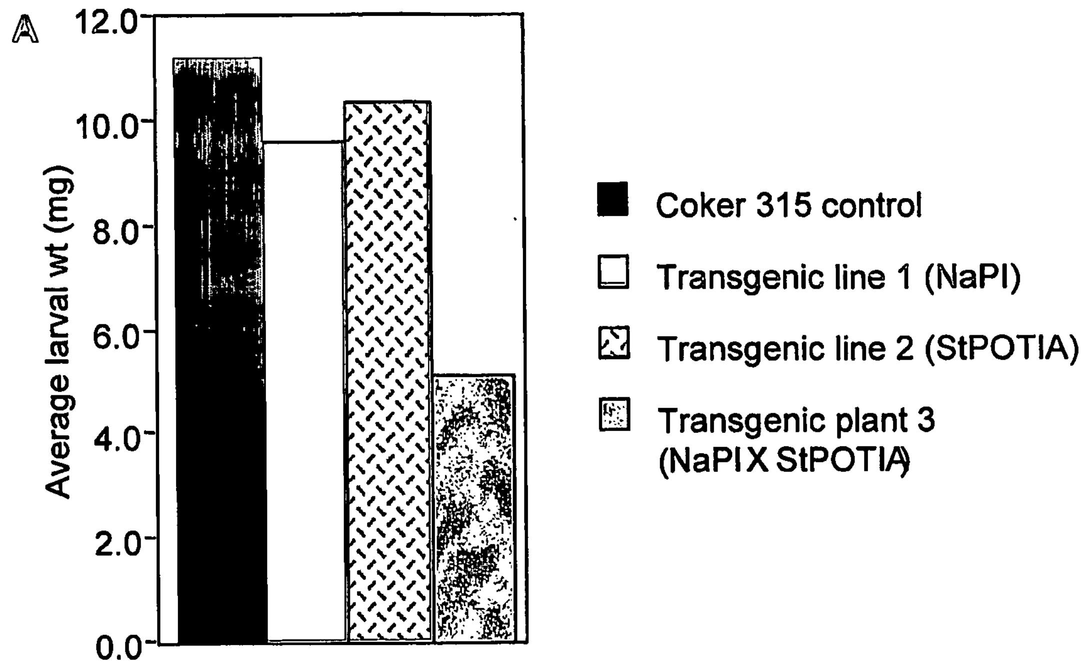

Figure 27A

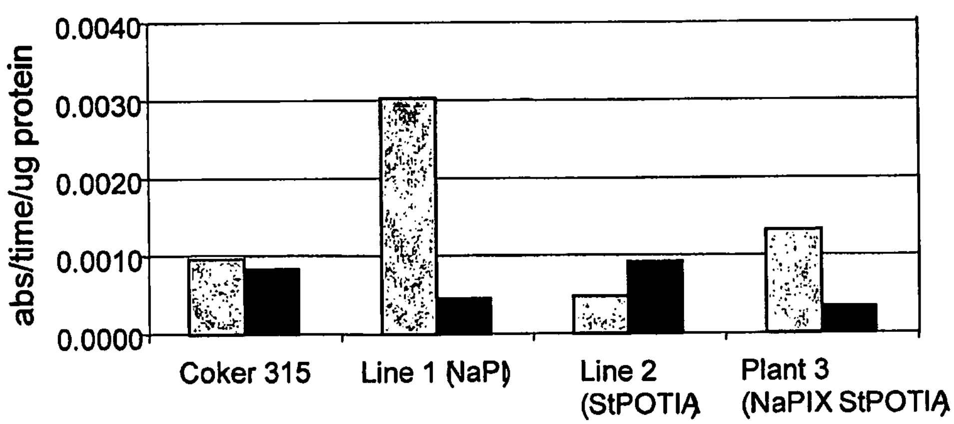

Figure 27B

10/554237

```
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      M  K  L  L  A  V  T  L  L  A  F  A  A  V  V  S  A  R

ggatccATGAAACTCTTGGCTGTGACTCTATTGGCtttcg
FwBacRECH2 Primer---------------------->
                                TTGGCTTTCGCCGCGGTCGTCTCCGCGAGG
                                FwBacRECH2 Primer-------------

Aacgg [A] tcccaccatcaccatcaccatGTTCACCTCGAGGATTCTATTGATCTGGAAGAT     120
 N  G  S  H  H  H  H  H  H  V  H  L  E  D  S  I  D  L  E  D
Aacgg [G] tccc
--------->

ATTACCGCTTGGGGATACCTCACCAAATTCGGTATTCCAGAAGCTGAGAAAATCCGCAAC     180
I  T  A  W  G  Y  L  T  K  F  G  I  P  E  A  E  K  I  R  N

GCTGAAGAAGCTAGCTCTGCTAGCAGGATCGTCGGTGGTTCATTGTCCAGTGTCGGACAG     240
 A  E  E  A  S  S  A  S  R  I  V  G  G  S  L  S  S  V  G  Q

ATCCCTTACCAGGCTGGTCTCGTCATTGACTTAGCAGGTGGCCAGGCTGTCTGCGGAGGC     300
 I  P  Y  Q  A  G  L  V  I  D  L  A  G  G  Q  A  V  C  G  G

TCCCTGATCAGCGCTTCCCGCGTACTGACCGCTGCTCACTGCTGGTTCGACGGCCAAAAC     360
 S  L  I  S  A  S  R  V  L  T  A  A  H  C  W  F  D  G  Q  N

CAGGCCTGGAGATTCACCGTTGTTCTTGGTTCCACCACCTTGTTCTCTGGCGGTACCAGA     420
 Q  A  W  R  F  T  V  V  L  G  S  T  T  L  F  S  G  G  T  R

ATCCCTACATCCAATGTTGTTATGCACGGAAGCTGGACTCCTAGCCTTATCCGTAACGAT     480
 I  P  T  S  N  V  V  M  H  G  S  W  T  P  S  L  I  R  N  D

GTTGCCGTAATCAGATTGGGCACCAACGTAGCAACCTCAAACACCATTGCCATCATCGCT     540
 V  A  V  I  R  L  G  T  N  V  A  T  S  N  T  I  A  I  I  A

CTACCCAGCGGCAGCCAGATCAACGAGAACTTCGCCGGTGAAACCGCCCTCGCCTCCGGC     600
 L  P  S  G  S  Q  I  N  E  N  F  A  G  E  T  A  L  A  S  G

TTCGGTCTCACCAGTGACACCGGCAGCATCTCCAGCAACCAGGCTCTGAGCCACGTCAAC     660
 F  G  L  T  S  D  T  G  S  I  S  S  N  Q  A  L  S  H  V  N

CTGCCAGTGATCACCAACGCTGTGTGCAGAAATTCATTCCCCCTGCTGATCCAGGACTCT     720
 L  P  V  I  T  N  A  V  C  R  N  S  F  P  L  L  I  Q  D  S

AACATTTGCACCAGCGGTGCCAACGGCAGGAGCACTTGCCGCGGTGACTCCGGCGGTCCT     780
 N  I  C  T  S  G  A  N  G  R  S  T  C  R  G  D  S  G  G  P

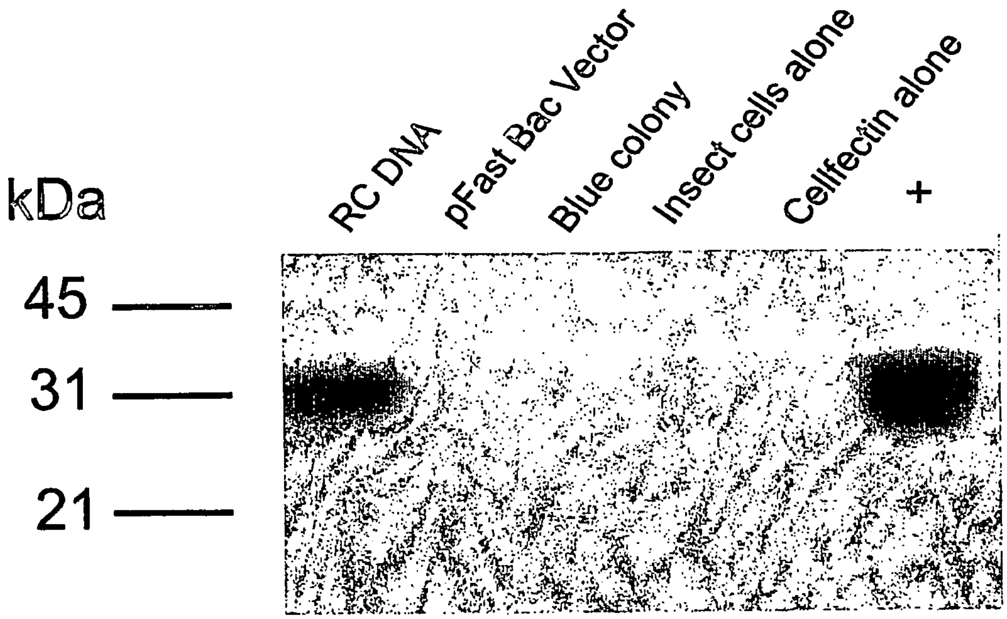
CTCGTCGTCACCAGGAACAACAGACCACTCTTGATCGGTATCACCTCTTTCGGATCTGCC     840
 L  V  V  T  R  N  N  R  P  L  L  I  G  I  T  S  F  G  S  A

CGCGGTTGCCAAGTTGGATCTCCCGCTGCCTTCGCCAGAGTCACCTCTTACATCAGCTG      900
 R  G  C  Q  V  G  S  P  A  A  F  A  R  V  T  S  Y  I  S  W

GATCAACGGCCAGCTCTAAaagctt                                        925
 I  N  G  Q  L  *  K  L

GATCAACGGCCAGCTCTAAaagctt
<-----------RvRECH Primer
```

Figure 28



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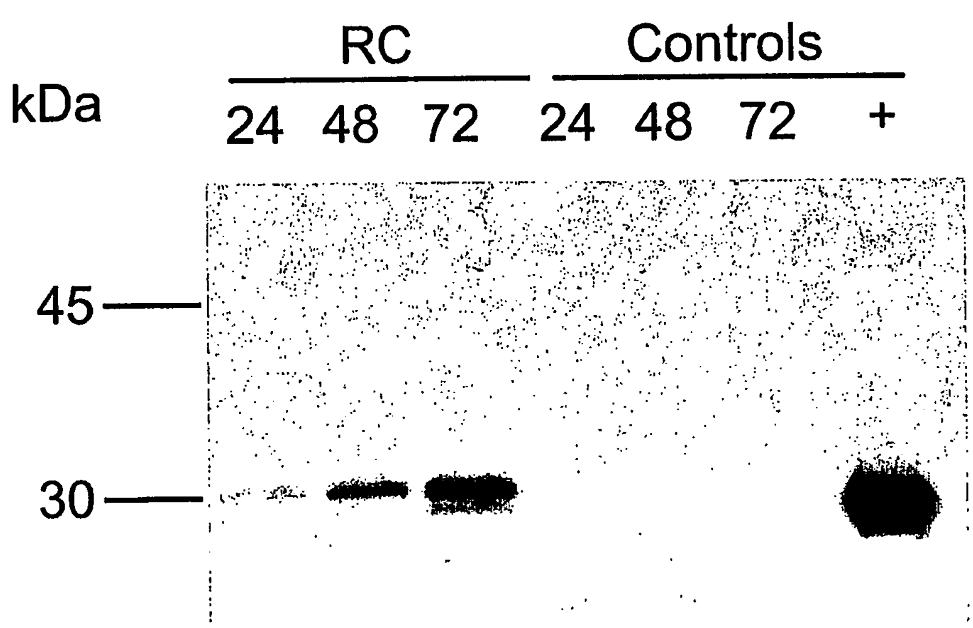

Figure 29B

SEQUENCE LISTING

<110> Hexima Limited
La Trobe University
Dunse, Kerry, Michelle (US ONLY)
Heath, Robyn, Louise (US ONLY)
Anderson, Marilyn, Anne (US ONLY)

<120> Novel insect enzymes and inhibitors thereof

<130> 12440340/EJH

<150> US 60/465,054
<151> 2003-04-23

<160> 93

<170> PatentIn version 3.1

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20 25 30

Leu Ile Ser Ala Ser Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Gln Asn Gln Ala Trp Arg Phe Thr Val Val Leu Val Met His Gly
50 55 60

Ser Trp Thr Pro Ser Leu Ile Arg Asn Asp Val Ala Val Ile Arg Leu
65 70 75 80

Gly Thr Asn Val Ala Thr Ser Asn Thr Ile Ala Ile Ile Ala Leu Pro
85 90 95

Ser Gly Ser Gln Ile Asn Glu Asn Phe Ala Gly Glu Thr Ala Leu Ala
100 105 110

Ser Gly Phe Gly Leu Thr Ser Asp Thr Gly Ser Ile Ser Ser Asn Gln
115 120 125

Ala Leu Ser His Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Arg
130 135 140

Asn Ser Phe Pro Leu Leu Ile Gln Asp Ser Asn Ile Cys Thr Ser Gly
145 150 155 160

Ala Asn Gly Arg Ser Thr Cys Arg Gly Asp Ser Gly Gly Pro Leu Val
165 170 175

Val Thr Arg Asn Asn Arg Pro Leu Leu Ile Gly Ile Thr Ser Phe Gly
180 185 190

Ser Ala Arg Gly Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val
195 200 205

Thr Ser Tyr Ile Ser Trp Ile Asn Gly Gln Leu
210 215

<210> 3
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<213> Helicoverpa

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Val His Leu Glu Asp Ser Ile Asp Leu Glu Asp Ile Thr Ala Trp Gly
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Tyr Leu Thr Lys Phe Gly Ile Pro Glu Ala Glu Lys Ile Arg Asn Ala
20 25 30

Glu Glu Ala Ser Ser Ala Ser Arg
35 40

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accgctgctc actgctggtt cgacggccaa aaccaggcct ggagattcac cgttgttctt 180

ggttccacca ccttgttctc tggcggtacc agaatcccta catccaatgt tgttatgcac 240

ggaagctgga ctcctagcct tatccgtaac gatgttgccg taatcagatt gggcaccaac 300

gtagcaacct caaacaccat tgccatcatc gctctaccca gcggcagcca gatcaacgag 360

aacttcgccg gtgaaaccgc cctcgcctcc ggcttcggtc tcaccagtga caccggcagc 420

atctccagca accaggctct gagccacgtc aacctgccag tgatcaccaa cgctgtgtgc 480

agaaattcat tccccctgct gatccaggac tctaacattt gcaccagcgg tgccaacggc 540

aggagcactt gccgcggtga ctccggcggt cctctcgtcg tcaccaggaa caacagacca 600

ctcttgatcg gtatcacctc tttcggatct gcccgcggtt gccaagttgg atctcccgct 660

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atcgtcggtg gttcattgtc cagtgtcgga cagatccctt accaggctgg tctcgtcatt 180

gacttagcag gtggccaggc tgtctgcgga ggctccctga tcagcgcttc ccgcgtactg 240

accgctgctc actgctggtt cgacggccaa aaccaggcct ggagattcac cgttgttctt 300

ggttccacca ccttgttctc tggcggtacc agaatcccta catccaatgt tgttatgcac 360

ggaagctgga ctcctagcct tatccgtaac gatgttgccg taatcagatt gggcaccaac 420

gtagcaacct caaacaccat tgccatcatc gctctaccca gcggcagcca gatcaacgag 480

aacttcgccg gtgaaaccgc cctcgcctcc ggcttcggtc tcaccagtga caccggcagc 540

atctccagca accaggctct gagccacgtc aacctgccag tgatcaccaa cgctgtgtgc 600

agaaattcat tccccctgct gatccaggac tctaacattt gcaccagcgg tgccaacggc 660

aggagcactt gccgcggtga ctccggcggt cctctcgtcg tcaccaggaa caacagacca 720

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gccttcgcca gagtcacctc ttacatcagc tggatcaacg gccagctcta aaatatcgaa 840

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20 25 30

Leu Ile Ser Ala
35

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<220>
<223> FWBacRECH2 (5'-3') primer

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20 25 30

Thr Leu Asn Cys Asp Pro Arg Ile Ala Tyr Gly Val Cys Pro Arg Ser
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Glu Glu Lys Lys Asn
50

<210> 23
<211> 58
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<400> 23

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Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Arg
20 25 30

Asn Pro Lys Ala Cys Thr Leu Asn Cys Asp Pro Arg Ile Ala Tyr Gly
35 40 45

Val Cys Pro Arg Ser Glu Glu Lys Lys Asn
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1 5 10 15

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20 25 30

Asn Pro Lys Ala Cys Pro Arg Asn Cys Asp Pro Arg Ile Ala Tyr Gly
35 40 45

Ile Cys Pro Leu Ala Glu Glu Lys Lys Asn
50 55

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1 5 10 15

Phe Ser Asp Asp Gly Thr Phe Val Cys Glu Gly Glu Ser Asp Pro Lys
20 25 30

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35 40 45

Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn
50 55

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1 5 10 15

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20 25 30

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35 40 45

Ile Cys Pro Leu Ser Glu Glu Lys Lys Asn
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Phe Ser Asp Asp Gly Thr Phe Ile Cys Glu Gly Glu Ser Glu Tyr Ala
            20   25   30

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        35   40   45

Ser Lys Val Ala Val Ser
    50

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<213> Helicoverpa

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1   5   10   15

Gly Leu Val Ile Asp Leu Ala Gly Gly Gln Ala Val Cys Gly Gly Ser
            20   25   30

Leu Ile Ser Ala
35

<210> 29
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<213> Helicoverpa

<400> 29

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1 5 10 15

Gly Leu Val Ile Asp Phe Ala Gly Gly Gln Ala Val Cys
20 25

<210> 30
<211> 29
<212> PRT
<213> Helicoverpa

<400> 30

Ile Val Gly Gly Ser Thr Ser Ser Val Gly Gln Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ala Ser Phe Ala Gly Gly Gln Ala Val Cys
20 25

<210> 31
<211> 29
<212> PRT
<213> Helicoverpa

<400> 31

Ile Val Gly Gly Ser Val Thr Thr Leu Asp Ala Tyr Pro Thr Ile Ala
1 5 10 15

Gly Leu Val Tyr Asn Phe Ala Gly Gly Gln Ala Val Cys
20 25

<210> 32
<211> 295
<212> PRT
<213> H. punctigera

<400> 32

Met Lys Leu Leu Ala Val Thr Leu Leu Ala Phe Ala Ala Val Val Ser
1 5 10 15

Ala Arg Asn Ile Asp Leu Glu Asp Val Ile Asp Leu Glu Asp Ile Thr
20 25 30

Ala Tyr Asp Tyr His Thr Lys Ile Gly Ile Pro Leu Ala Glu Lys Ile
35 40 45

Arg Ala Ala Glu Glu Glu Ala Glu Arg Asn Pro Ser Arg Ile Val Gly
50 55 60

Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala Gly Leu Leu
65 70 75 80

Ala Thr Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser Leu Leu Asn
85 90 95

Asn Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn
100 105 110

Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg Leu Phe Ser
115 120 125

Gly Gly Thr Arg Leu Asn Thr Ala Ser Val Val Met His Gly Ser Trp
130 135 140

Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Met Ile Asn Leu Pro Ser
145 150 155 160

Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly
165 170 175

Asn Glu Leu Asn Asn Asn Phe Asn Gly Ala Thr Ala Val Ala Ser Gly
180 185 190

Phe Gly Leu Ala Arg Asp Gly Gly Ser Val Asp Gly Asn Leu Arg His
195 200 205

Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Thr Val Ser Phe Pro
210 215 220

Gly Ile Ile Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg
225 230 235 240

Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val Thr Ser Asn
245 250 255

Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Ala Arg Gly
260 265 270

Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Ile
275 280 285

Ser Trp Ile Asn Gln Arg Leu
290 295

<210> 33
<211> 292
<212> PRT
<213> H. punctigera

<400> 33

Leu Ala Val Thr Leu Leu Ala Phe Ala Ala Val Val Ser Ala Arg Asn
1 5 10 15

Ile Asp Leu Glu Asp Val Ile Asp Leu Glu Asp Ile Thr Ala Tyr Asp
20 25 30

Tyr His Thr Lys Ile Gly Ile Pro Leu Ala Glu Lys Ile Arg Ala Ala

35 40 45

Glu Glu Glu Ala Glu Arg Asn Pro Ser Arg Ile Val Gly Gly Ser Thr
50 55 60

Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala Gly Leu Leu Ala Thr Phe
65 70 75 80

Ala Ser Gly Gln Gly Val Cys Gly Gly Ser Leu Leu Asn Asn Arg Arg
85 90 95

Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn Gln Ala Arg
100 105 110

Ser Phe Thr Val Val Leu Gly Ser Val Arg Leu Phe Ser Gly Gly Thr
115 120 125

Arg Leu Asn Thr Ala Ser Val Val Met His Gly Ser Trp Asn Pro Asn
130 135 140

Leu Ile Arg Asn Asp Ile Ala Met Ile Asn Leu Pro Ser Asn Val Ala
145 150 155 160

Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly Asn Glu Leu
165 170 175

Asn Asn Asn Phe Asn Gly Ala Thr Ala Val Ala Ser Gly Phe Gly Leu
180 185 190

```
Ala Arg Asp Gly Gly Ser Val Asp Gly Asn Leu Arg His Val Asn Leu
        195                 200                 205

Pro Val Ile Thr Asn Ala Val Cys Thr Val Ser Phe Pro Gly Ile Ile
    210                 215                 220

Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg Gly Thr Cys
225                 230                 235                 240

Gln Gly Asp Ser Gly Gly Pro Leu Val Val Thr Ser Asn Asn Arg Arg
                245                 250                 255

Ile Leu Ile Gly Val Thr Pro Phe Gly Ser Ala Arg Gly Cys Gln Val
            260                 265                 270

Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Ile Ser Trp Ile
        275                 280                 285

Asn Gln Arg Leu
    290
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<210> 34
<211> 295
<212> PRT
<213> H. punctigera

<400> 34

Met Lys Leu Leu Ala Val Thr Leu Leu Ala Phe Ala Ala Ile Val Ser
1 5 10 15

Ala Arg Asn Ile Asp Leu Glu Asp Val Ile Asp Leu Glu Asp Ile Thr
20 25 30

Ala Tyr Asp Tyr His Thr Lys Ile Gly Ile Pro Leu Ala Glu Lys Ile
35 40 45

Arg Ala Ala Glu Glu Glu Ala Glu Arg Asn Pro Ser Arg Ile Val Gly
50 55 60

Gly Ser Ile Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala Gly Leu Leu
65 70 75 80

Ala Thr Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser Leu Leu Asn
85 90 95

Asn Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn
100 105 110

Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg Leu Phe Ser
115 120 125

Gly Gly Thr Arg Leu Asn Thr Ala Ser Val Val Met His Gly Ser Trp
130 135 140

Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Ile Ile Asn Leu Pro Ser
145 150 155 160

Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly
165 170 175

Asn Glu Leu Asn Asn Asn Phe Asn Gly Ala Thr Ala Val Ala Ser Gly
180 185 190

Phe Gly Leu Ala Asn Asp Gly Gly Ser Val Asp Gly Asn Leu Arg His
195 200 205

Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Thr Val Ser Phe Pro
210 215 220

Gly Ile Ile Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg
225 230 235 240

Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val Thr Ser Asn
245 250 255

Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Ala Arg Gly
260 265 270

Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Ile
275 280 285

Ser Trp Ile Asn Asn Leu Leu
290 295

<210> 35
<211> 276
<212> PRT
<213> H. punctigera

<400> 35

Ile Asn His Glu Ala Val Val Asp Leu Glu Asp Ile Thr Ala Tyr Gly
1 5 10 15

Tyr His Thr Lys Val Gly Ile Pro Leu Ala Glu Glu Ile Arg Ile Ala
20 25 30

Glu Leu Glu Ala Ser Arg Asn Pro Ser Arg Ile Val Gly Gly Ser Ser
35 40 45

Ala Ser Leu Gly Gln Phe Pro Tyr Gln Ala Gly Leu Leu Ile Asn Leu
50 55 60

Pro Leu Gly Gln Ser Val Cys Gly Gly Ser Leu Leu Asn Gln Arg Arg
65 70 75 80

Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn Gln Ala Asn
85 90 95

Ser Leu Thr Val Ile Leu Gly Ser Ile Asn Leu Tyr Phe Gly Gly Thr
100 105 110

Arg Leu Asn Ser Asn Ser Val Val Met His Gly Ser Trp Asn Pro Asn
115 120 125

Leu Ile Arg Asn Asp Ile Ala Ile Ile Asn Leu Pro Ser Asn Val Gly
130 135 140

Thr Ser Asn Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly Asn Glu Leu
145 150 155 160

Asn Asn Gln Phe Ala Gly Phe Thr Ala Thr Ala Ser Gly Phe Gly Arg
165 170 175

Thr Arg Asp Gly Gly Ser Val Ser Pro Thr Leu Asn His Val Asn Leu
180 185 190

Pro Val Ile Thr Asn Asn Val Cys Trp Gln Ser Phe Pro Leu Tyr Ile
195 200 205

Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg Ser Thr Cys
210 215 220

Gln Gly Asp Ser Gly Gly Pro Leu Val Val Thr Ser Asn Asn Arg Arg
225 230 235 240

Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Asp Arg Gly Cys Gln Val
245 250 255

Gly Ala Pro Ala Ala Phe Ala Arg Val Thr Ser Tyr Ile Ser Trp Ile
260 265 270

Asn Gln Arg Leu
275

<210> 36
<211> 292
<212> PRT
<213> H. punctigera

<400> 36

Met Lys Leu Phe Leu Gly Val Cys Leu Thr Leu Ala Val Ala Val Ser
1 5 10 15

Ala Val Glu Ile Ala Thr Pro Asp Ala Asp Ser Pro Val Phe Gly Tyr
20 25 30

His Ala Lys Phe Gly Ile Ala Glu Ala Ala Arg Ile Lys Ser Ala Glu
35 40 45

Glu Val Gln Ser Phe Asn Gly Gln Arg Ile Val Gly Gly Ser Ile Thr
50 55 60

Asn Ile Ala Asn Val Pro Tyr Gln Ala Gly Leu Val Ile Thr Ile Phe
65 70 75 80

Ile Phe Gln Ser Val Cys Gly Ala Ser Leu Ile Ser His Asn Arg Leu
85 90 95

Val Thr Ala Ala His Cys Lys Ser Asp Gly Val Leu Thr Ala Asn Ser
100 105 110

Phe Thr Val Val Leu Gly Ser Asn Thr Leu Phe Phe Gly Gly Thr Arg
115 120 125

Ile Asn Thr Asn Asp Val Val Met His Pro Asn Trp Asn Pro Asn Thr
130 135 140

Ala Ala Asn Asp Ile Ala Val Leu Arg Ile Ser Ser Val Ser Phe Ser
145 150 155 160

Asn Val Ile Gln Pro Ile Ala Leu Pro Ser Gly Asp Glu Leu Asn Asn
165 170 175

Leu Phe Val Gly Ala Asn Ala Leu Ala Ser Gly Phe Gly Arg Thr Ser
180 185 190

Asp Ser Gly Ser Ile Gly Thr Asn Gln Gln Leu Ser Ser Val Thr Ile
195 200 205

Pro Val Ile Thr Asn Ala Gln Cys Ala Ala Val Tyr Gly Ser Gly Phe
210 215 220

Val His Ala Ser Asn Ile Cys Thr Ser Gly Ala Gly Gly Lys Gly Thr
225 230 235 240

Cys Asn Gly Asp Ser Gly Gly Pro Leu Ala Val Asp Ser Asn Asn Arg
245 250 255

Lys Ile Leu Ile Gly Val Thr Ser Tyr Gly Ala Gln Ala Gly Cys Ala
260 265 270

Ala Gly Phe Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Val Asp Trp
275 280 285

Val Gln Ser Gln
290

<210> 37
<211> 257
<212> PRT
<213> H. punctigera

<400> 37

His Asn Lys Trp Val Leu Thr Ala Ala His Cys Leu Ala Asn Arg Ile
1 5 10 15

Thr Phe Val Val Arg Phe Gly Leu Thr Asn Leu Thr Arg Pro Glu Ile
20 25 30

Leu Val Glu Ser Ala Asn Lys Tyr Ile His Pro Asp Tyr Asp Glu Ile
35 40 45

Arg Ala Gly Val Gln Thr Ala Asp Leu Ala Leu Val Gly Leu Asp His
50 55 60

His Ile Glu Tyr Ser Ala Asn Val Gln Pro Ser Arg Leu Met Ser Ser
65 70 75 80

Ala Gln Lys Asn Ile Asn Tyr Glu Gly Ile Gln Met Ile Val Ser Gly
85 90 95

Phe Gly Arg Thr Asp Asp Leu Trp Asn Gly Gly Ala Ala Ser Glu Ile
100 105 110

Leu Leu Trp Val Tyr Gln Arg Gly Val Ser Asn Glu Glu Cys Leu Arg
115 120 125

Trp Tyr Pro Thr Ser Gln Val Ile Lys Glu Glu Thr Ile Cys Ala Gly
130 135 140

Tyr Trp Asp Asn Pro Ser Gln Ser Ser Cys Gln Gly Asp Ser Gly Gly
145 150 155 160

Pro Leu Thr Ile Ile Asp Ala Asp Gly Glu Arg Thr Gln Val Gly Ile
165 170 175

Val Ser Phe Gly Ser Thr Ala Gly Cys Asn Ser Pro Phe Pro Ser Gly
180 185 190

Tyr Val Arg Pro Gly His Tyr His Asp Trp Phe Thr Glu Val Thr Gly
        195   200   205

Ile Asn Phe Asp Trp Asp Ser Asp Ala Ile Ile Pro Gly Ser Ser Glu
    210   215   220

Ser Glu Glu Asp Gly Ser Asn Pro Ser Ser Glu Glu Asp Ala Gly Ser
225   230   235   240

Pro Pro Ser Glu Glu Glu Glu Ala Pro Glu Lys Val Arg Val Val Glu
                245   250   255

Tyr

<210> 38
<211> 19
<212> DNA
<213> artificial sequence

<220>
<223> FWG1 primer

<400> 38
tcccttacca ggcgctgtc 19

<210> 39
<211> 18
<212> DNA
<213> artificial sequence

<220>
<223> RVG4 primer

<400> 39
tctggcgaag gcagcagg 18

<210> 40
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> Y79Fw primer

<400> 40
ctgctagcct cggacaattc 20

<210> 41
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> Y72Fw primer

<400> 41
ctggagtgca gactgctgac 20

<210> 42
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> Y72Rv primer

<400> 42
ggatgatggc gtcgctgtcc 20

<210> 43
<211> 213
<212> PRT
<213> H. punctigera

<400> 43

Pro Tyr Gln Ala Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val
1 5 10 15

Cys Gly Ala Ser Leu Ile Pro His Asn Arg Leu Val Thr Ala Ala His
20 25 30

Cys Lys Ser Asp Gly Val Leu Thr Ala Asn Ser Phe Thr Val Val Leu
35 40 45

Gly Ser Asn Thr Leu Phe Phe Gly Gly Thr Arg Ile Asn Thr Asn Asp
50 55 60

Val Val Met His Pro Asn Trp Asn Pro Ser Thr Ala Ala Asn Asp Ile
65 70 75 80

Ala Val Met Arg Ile Ser Ser Val Ser Phe Ser Asn Val Ile Gln Pro
85 90 95

Ile Ala Leu Pro Ser Gly Asp Glu Leu Asn Asn Leu Phe Val Gly Ala
100 105 110

Asn Ala Leu Ala Ser Gly Phe Gly Arg Thr Ser Asp Gly Gly Ser Ile
115 120 125

Gly Ser Asn Gln Gln Val Ser Ser Val Thr Ile Pro Val Ile Thr Asn
130 135 140

Asp Glu Cys Ala Ala Val Tyr Gly Ser Ala Phe Val His Ser Ser Asn
145 150 155 160

Ile Cys Thr Ser Gly Ala Gly Gly Lys Gly Thr Cys Asn Gly Asp Ser
165 170 175

Gly Gly Pro Leu Ala Ile Asp Ser Asn Asn Glu Lys Ile Leu Ile Gly
180 185 190

Val Thr Ser Tyr Gly Ala Gln Ala Gly Cys Ala Ala Gly Leu Pro Ala
195 200 205

Ala Phe Ala Arg Lys
210

<210> 44
<211> 213
<212> PRT
<213> H. punctigera

<400> 44

Pro Tyr Gln Ala Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val
1 5 10 15

Cys Gly Ala Ser Leu Ile Ser His Asn Arg Leu Val Thr Ala Ala His
20 25 30

Cys Lys Phe Asp Gly Val Met Thr Ala Asn Ser Phe Thr Val Val Leu
35 40 45

Gly Ser Asn Thr Leu Phe Phe Gly Gly Thr Arg Ile Asn Thr Asn Asp
50 55 60

Val Val Met His Pro Asn Trp Asn Pro Ser Thr Val Ala Asn Asp Ile
65 70 75 80

Ala Val Ile Arg Ile Ser Ser Ile Val Phe Asn Asn Val Ile Gln Pro
85 90 95

Ile Ala Leu Pro Ser Gly Asp Glu Leu Asn Asn Leu Phe Val Gly Ala
100 105 110

Asn Ala Leu Ala Ser Gly Phe Gly Arg Thr Ser Asp Ser Gly Gly Ile
115 120 125

Gly Thr Asn Gln Gln Leu Ser Ser Val Thr Ile Pro Val Ile Thr Asn
    130   135   140

Ala Glu Cys Ala Ala Val Tyr Gly Pro Ala Phe Val His Asp Thr Asn
145   150   155   160

Ile Cys Thr Ser Gly Ala Gly Gly Lys Gly Thr Cys Asn Gly Asp Ser
                165   170   175

Gly Gly Pro Leu Ala Val Asp Ser Asn Asp Lys Lys Ile Leu Ile Gly
            180   185   190

Val Thr Ser Tyr Gly Ala Ala Asp Gly Cys Ala Ala Gly Phe Pro Ala
        195   200   205

Ala Ser Pro Glu Arg
    210

<210> 45
<211> 177
<212> PRT
<213> H. punctigera

<400> 45

Pro Tyr Gln Ala Gly Leu Leu Ala Asn Phe Ala Ser Gly Gln Gly Val
1   5   10   15

Cys Gly Gly Ser Leu Leu Asn Gln Arg Arg Val Leu Thr Ala Ala His
            20   25   30

Cys Trp Phe Asp Gly Arg Asn Gln Ala Arg Ser Phe Thr Val Val Leu
35 40 45

Gly Ser Val Arg Leu Phe Ser Gly Gly Thr Arg Leu Asp Thr Ala Ser
50 55 60

Val Val Met His Gly Ser Trp Asn Pro Asn Leu Ile Arg Asn Asp Ile
65 70 75 80

Ala Met Ile Asn Leu Pro Ser Asn Val Ala Thr Ser Gly Asn Ile Ala
85 90 95

Pro Ile Ala Leu Pro Ser Gly Asn Glu Leu Asn Asn Asn Phe Asn Gly
100 105 110

Ala Thr Ala Thr Ala Ser Gly Phe Gly Leu Ala Arg Asp Gly Gly Ser
115 120 125

Val Asp Gly Asn Leu Arg His Val Asn Leu Pro Val Ile Thr Asn Ala
130 135 140

Val Cys Thr Val Ser Phe Pro Gly Ile Ile Gln Ser Ser Asn Ile Cys
145 150 155 160

Thr Ser Gly Ala Asn Gly Arg Ser Thr Cys Gln Gly Asp Ser Gly Gly
165 170 175

Pro

<210> 46
<211> 217
<212> PRT
<213> H. punctigera

<400> 46

Ser Ala Ser Leu Gly Gln Phe Pro Tyr Gln Ala Gly Leu Leu Ile Asn
1 5 10 15

Leu Pro Leu Gly Gln Ser Val Cys Gly Gly Ser Leu Leu Asn Gln Arg
20 25 30

Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn Gln Ala
35 40 45

Thr Ser Leu Thr Val Ile Leu Gly Ser Ile Asn Leu Phe Phe Gly Gly
50 55 60

Thr Arg Leu Asn Ser Asn Ser Val Val Met His Gly Ser Trp Asn Pro
65 70 75 80

Asn Leu Ile Arg Asn Asp Ile Ala Ile Ile Asn Leu Pro Ser Asn Val
85 90 95

```
Gly Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly Asn Glu
            100                 105                 110

Leu Asn Asn Gln Phe Ala Gly Phe Thr Ala Thr Ala Ser Gly Phe Gly
        115                 120                 125

Leu Thr Arg Asp Gly Gly Asn Val Ser Pro Thr Leu Asn His Val Asn
    130                 135                 140

Leu Pro Val Ile Thr Asn Asn Val Cys Trp Gln Ser Phe Pro Leu Tyr
145                 150                 155                 160

Ile Gln Ser Thr Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg Gly Thr
                165                 170                 175

Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val Thr Ser Asn Asn Arg
            180                 185                 190

Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Asp Arg Gly Cys Gln
        195                 200                 205

Val Gly Ala Pro Ala Ala Phe Ala Arg
    210                 215
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<210> 47
<211> 170
<212> PRT
<213> H. punctigera

<400> 47

Ser Gly Val Gln Thr Ala Asp Leu Ala Leu Val Gly Leu Asp Gln Glu
1 5 10 15

Ile Glu Tyr Ser Ala Asn Val Gln Pro Ser Arg Leu Met Ser Ser Ala
20 25 30

Gln Lys Asn Ile Asn Tyr Glu Gly Ile Gln Met Ile Val Ser Gly Phe
35 40 45

Gly Arg Thr Asp Asp Leu Trp Asn Gly Gly Ala Ala Ser Glu Ile Leu
50 55 60

Leu Trp Val Tyr Gln Arg Gly Val Ser Asn Glu Glu Cys Leu Arg Trp
65 70 75 80

Tyr Pro Thr Ser Gln Val Ile Lys Glu Gln Thr Ile Cys Ala Gly Tyr
85 90 95

Trp Asp Asn Pro Ser Gln Ser Ser Cys Gln Gly Asp Ser Gly Gly Pro
100 105 110

Leu Thr Ile Ile Asp Ala Asp Gly Glu Arg Thr Gln Val Gly Ile Val
115 120 125

Ser Phe Gly Ser Thr Ala Gly Cys Asn Ser Pro Phe Pro Ser Gly Tyr
130 135 140

Val Arg Pro Gly His Tyr His Asp Trp Phe Thr Glu Val Thr Gly Ile
145 150 155 160

Asn Phe Asp Trp Asp Ser Asp Ala Ile Ile
165 170

<210> 48
<211> 279
<212> PRT
<213> H. punctigera

<400> 48

Ala Val Ser Ala Val Glu Ile Gly Thr Pro Asp Ala Asp Ser Pro Val
1 5 10 15

Phe Gly Tyr His Ala Lys Phe Gly Ile Pro Glu Ala Ala Arg Ile Lys
20 25 30

Ser Ala Glu Glu Val Gln Ser Phe Asn Gly Gln Arg Ile Val Gly Gly
35 40 45

Ser Ile Thr Asp Ile Ala Asn Val Pro Tyr Gln Ala Gly Leu Val Ile
50 55 60

Thr Ile Phe Ile Phe Gln Ser Val Cys Gly Ala Ser Leu Ile Ser His
65 70 75 80

Asn Arg Leu Val Thr Ala Ala His Cys Lys Ser Asp Gly Val Leu Thr
85 90 95

Ala Asn Ser Phe Thr Val Val Leu Gly Ser Asn Thr Leu Phe Phe Gly
100 105 110

Gly Thr Arg Ile Asn Thr Asn Asp Val Val Met His Pro Asn Trp Asn
115 120 125

Pro Ser Thr Ala Ala Asn Asp Ile Ala Val Met Arg Ile Ser Ser Val
130 135 140

Ser Phe Ser Asn Val Ile Gln Pro Ile Ala Leu Pro Ser Gly Asp Glu
145 150 155 160

Leu Asn Asn Leu Phe Val Gly Ala Asn Ala Leu Ala Ser Gly Phe Gly
165 170 175

Arg Thr Ser Asp Gly Gly Ser Ile Gly Ser Asn Gln Gln Val Ser Ser
180 185 190

Val Thr Ile Pro Val Ile Thr Asn Asp Glu Cys Ala Ala Val Tyr Gly
195 200 205

Ser Ala Phe Val His Ser Ser Asn Ile Cys Thr Ser Gly Ala Gly Gly
210 215 220

Lys Gly Thr Cys Asn Gly Asp Ser Gly Gly Pro Leu Ala Val Asp Ser
225 230 235 240

Asn Asn Glu Lys Ile Leu Ile Gly Val Thr Ser Tyr Gly Ala Gln Ala
245 250 255

Gly Cys Ala Val Gly Leu Pro Ala Ala Phe Ala Arg Val Thr Ser Phe
260 265 270

Val Ser Trp Val Gln Ser Gln
275

<210> 49
<211> 292
<212> PRT
<213> H. punctigera

<400> 49

Met Lys Leu Phe Leu Gly Val Cys Leu Ala Leu Ala Val Ala Val Ser
1 5 10 15

Ala Val Glu Ile Gly Thr Pro Glu Ala Gly Ser Pro Val Phe Gly Tyr
20 25 30

His Ala Lys Phe Gly Ile Ala Glu Ala Ala Arg Ile Lys Ser Ala Glu
35 40 45

Glu Val Gln Ser Phe Asn Gly Gln Arg Ile Val Gly Gly Ser Ile Thr
50 55 60

Asn Ile Ala Asn Val Pro Tyr Gln Ala Gly Leu Val Ile Thr Ile Phe
65 70 75 80

Ile Phe Gln Ser Val Cys Gly Ala Ser Leu Ile Ser His Asn Arg Leu
85 90 95

Val Thr Ala Ala His Cys Lys Phe Asp Gly Val Met Thr Ala Asn Ser
100 105 110

Phe Thr Val Val Leu Gly Ser Asn Thr Leu Phe Phe Gly Gly Thr Arg
115 120 125

Ile Asn Thr Asn Asp Val Val Met His Pro Asn Trp Asn Pro Ser Thr
130 135 140

Val Ala Asn Asp Ile Ala Val Ile Arg Ile Ser Ser Ile Val Tyr Asn
145 150 155 160

Asn Val Ile Gln Pro Ile Ala Leu Pro Ser Gly Asp Glu Leu Asp Asn
165 170 175

Leu Phe Val Gly Ala Asn Ala Leu Ala Ser Gly Phe Gly Arg Thr Ser
180 185 190

Asp Ser Gly Gly Ile Gly Thr Asn Gln Gln Leu Ser Ser Val Thr Ile
195 200 205

Pro Val Ile Thr Asn Ala Glu Cys Ala Ala Val Tyr Gly Pro Ala Phe
210 215 220

Val His Asp Thr Asn Ile Cys Thr Ser Gly Ala Gly Gly Lys Gly Thr
225 230 235 240

Cys Asn Gly Asp Ser Gly Gly Pro Leu Ala Val Asp Ser Asn Asp Lys
245 250 255

Lys Ile Leu Ile Gly Val Thr Ser Tyr Gly Ala Ala Asp Gly Cys Ala
260 265 270

Ala Gly Phe Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Val Ser Trp
275 280 285

Val Gln Ser Gln
290

<210> 50
<211> 295
<212> PRT
<213> H. punctigera

<400> 50

Met Lys Leu Leu Ala Val Thr Leu Leu Ala Phe Ala Ala Val Val Ser
1 5 10 15

Ala Arg Asn Ile Asp Leu Glu Asp Val Ile Asp Leu Glu Asp Ile Thr
20 25 30

Ala Tyr Asp Tyr His Thr Lys Ile Gly Ile Pro Leu Ala Glu Glu Ile
35 40 45

Arg Ala Ala Glu Glu Glu Ala Glu Arg Asp Pro Ser Arg Ile Val Gly
50 55 60

Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala Gly Leu Leu
65 70 75 80

Ala Asn Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser Leu Leu Asn
85 90 95

Gln Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn
100 105 110

Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg Leu Phe Ser
115 120 125

Gly Gly Thr Arg Leu Asp Thr Ala Ser Val Val Met His Gly Ser Trp
130 135 140

Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Met Ile Asn Leu Pro Ser
145 150 155 160

Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly
165 170 175

Asn Glu Leu Asn Asn Asn Phe Asn Gly Ala Thr Ala Thr Ala Ser Gly
180 185 190

Phe Gly Leu Ala Arg Asp Gly Gly Ser Val Asp Gly Asn Leu Arg His
195 200 205

Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Thr Val Ser Phe Pro
210 215 220

Gly Ile Ile Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg
225 230 235 240

Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val Asn Ser Asn
245 250 255

Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Ala Arg Gly
260 265 270

Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Phe Ile
275 280 285

Ser Trp Ile Asn Gln Arg Leu
290 295

<210> 51
<211> 295
<212> PRT
<213> H. punctigera

<400> 51

Met Lys Leu Leu Ala Val Thr Leu Leu Ala Phe Ala Ala Val Val Ser
1 5 10 15

Ala Arg Asn Ile Asp Leu Glu Asp Val Ile Asp Leu Glu Asp Ile Thr
20 25 30

Ala Tyr Asp Tyr His Thr Lys Ile Gly Ile Pro Leu Ala Glu Lys Ile
35 40 45

Arg Ala Ala Glu Glu Glu Ala Glu Arg Asn Pro Ser Arg Ile Val Gly
50 55 60

Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala Gly Leu Leu
65 70 75 80

Ala Ser Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser Leu Leu Asn
85 90 95

Val Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly Arg Asn
100 105 110

Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg Leu Tyr Ser
115 120 125

```
Gly Gly Thr Arg Leu Asn Thr Ala Ser Val Val Met His Gly Ser Trp
    130                 135                 140

Asn Pro Asn Leu Val Arg Asn Asp Ile Ala Met Ile Asn Leu Pro Ser
145                 150                 155                 160

Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu Pro Ser Gly
                165                 170                 175

Asn Glu Leu Asn Asn Gln Phe Ala Gly Ala Thr Ala Thr Ala Ser Gly
            180                 185                 190

Phe Gly Leu Ala Arg Asp Gly Gly Val Ile Asp Gly Asn Leu Arg His
        195                 200                 205

Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Ser Gln Ser Phe Pro
    210                 215                 220

Gly Leu Ile Gln Ala Ser Asn Val Cys Thr Ser Gly Ala Asn Gly Arg
225                 230                 235                 240

Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val Asn Ser Asn
                245                 250                 255

Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser Ala Arg Gly
            260                 265                 270
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Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Ser Ser Tyr Ile
275 280 285

Ser Trp Ile Asn Gln Arg Leu
290 295

<210> 52
<211> 234
<212> PRT
<213> H. punctigera

<400> 52

Ile Val Gly Gly Ser Ser Ala Ser Leu Gly Gln Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ile Asn Leu Pro Leu Gly Gln Ser Val Cys Gly Gly Ser
20 25 30

Leu Leu Asn Gln Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Arg Asn Gln Ala Thr Ser Leu Thr Val Ile Leu Gly Ser Ile Asn
50 55 60

Leu Phe Phe Gly Gly Thr Arg Leu Asn Ser Asn Ser Val Val Met Gln
65 70 75 80

Gly Ser Trp Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Ile Ile Asn
85 90 95

Leu Pro Ser Asn Val Gly Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu
100 105 110

Pro Ser Gly Asn Glu Leu Asn Asn Gln Phe Ala Gly Phe Thr Ala Thr
115 120 125

Ala Ser Gly Phe Gly Leu Thr Arg Asp Gly Gly Asn Val Ser Pro Thr
130 135 140

Leu Asn His Val Asn Leu Pro Val Ile Thr Asn Asn Val Cys Trp Gln
145 150 155 160

Ser Phe Pro Leu Tyr Ile Gln Ser Thr Asn Ile Cys Thr Ser Gly Ala
165 170 175

Asn Gly Arg Gly Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val
180 185 190

Thr Ser Asn Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser
195 200 205

Asp Arg Gly Cys Gln Val Gly Ala Pro Ala Ala Phe Ala Arg Val Thr
210 215 220

Ser Tyr Ile Ser Trp Ile Asn Gln Arg Leu
225 230

<210> 53
<211> 296
<212> PRT
<213> H. punctigera

<400> 53

Met Ala Ala Ala Tyr Leu Leu Gly Leu Leu Phe Val Leu Gly Tyr Val
1 5 10 15

Gln Gly Gly Leu Leu Asn Ala Asp Pro Ala Ile Ile Glu Asp Leu Arg
20 25 30

Asp Ala Glu Phe Ser Ser Gly Ser Arg Ile Val Ala Gly Trp Pro Ala
35 40 45

Val Glu Gly Gln Ile Pro Tyr Gln Gly Ser Leu Arg Met Val Ser Ala
50 55 60

Ile Gly Gly Val Ser Ser Cys Gly Cys Ser Leu Ile His Asn Lys Trp
65 70 75 80

Val Leu Thr Ala Ala His Cys Leu Ala Asn Arg Ile Thr Phe Val Val
85 90 95

Arg Phe Gly Leu Thr Asn Leu Thr Arg Pro Glu Ile Leu Val Glu Ser
100 105 110

Thr Asn Lys Tyr Ile His Pro Glu Tyr Asp Glu Ile Arg Ala Gly Val
115 120 125

Gln Thr Ala Asp Leu Ala Leu Val Gly Leu Asp His Glu Ile Glu Tyr
130 135 140

Ser Ala Asn Val Gln Pro Ser Arg Leu Met Ser Ser Ala Gln Lys Asn
145 150 155 160

Ile Asn Tyr Glu Gly Ile Gln Met Ile Val Ser Gly Phe Gly Arg Thr
165 170 175

Asp Asp Leu Trp Asn Gly Gly Ala Ala Ser Glu Ile Leu Leu Trp Val
180 185 190

Tyr Gln Arg Gly Val Ser Asn Glu Glu Cys Leu Arg Trp Tyr Pro Thr
195 200 205

Ser Gln Val Ile Lys Glu Gln Thr Ile Cys Ala Gly Tyr Trp Asp Asn
210 215 220

Pro Ser Gln Ser Ser Cys Gln Gly Asp Ser Gly Gly Pro Leu Thr Ile
225 230 235 240

Ile Asp Ala Asp Gly Glu Arg Thr Gln Ser Arg Tyr Cys Glu Leu Arg
245 250 255

Ile His Cys Trp Asn Ala Ala His Ser Pro Gln Gly Tyr Val Arg Pro
260 265 270

Gly His Tyr His Asp Trp Phe Thr Glu Val Thr Gly Ile Asn Phe Asp
275 280 285

Trp Asp Ser Asp Ala Ile Ile Pro
290 295

<210> 54
<211> 365
<212> PRT
<213> H. punctigera

<400> 54

Met Ala Ala Ala Tyr Leu Leu Gly Leu Leu Phe Val Leu Gly Tyr Val
1 5 10 15

Gln Gly Gly Leu Leu Asn Ala Asp Pro Ala Ile Ile Glu Asp Leu Arg
20 25 30

Asp Ala Glu Phe Ser Ser Phe Ser Arg Ile Val Ala Gly Trp Pro Ala
35 40 45

Val Glu Gly Gln Ile Pro Tyr Gln Gly Ser Leu Arg Met Val Ser Ala
50 55 60

Ile Gly Gly Val Ser Ser Cys Gly Cys Ser Leu Ile His Asn Lys Trp
65 70 75 80

Val Leu Thr Ala Ala His Cys Leu Ala Asn Arg Ile Thr Phe Val Val
85 90 95

Arg Phe Gly Leu Thr Asn Leu Thr Arg Pro Glu Ile Leu Val Glu Ser
100 105 110

Thr Asn Lys Tyr Ile His Pro Glu Tyr Asp Glu Ile Arg Ala Gly Val
115 120 125

Gln Thr Ala Asp Leu Ala Leu Val Gly Leu Asp Gln Glu Ile Glu Tyr
130 135 140

Ser Ala Asn Val Gln Pro Ser Arg Leu Met Ser Ser Ala Gln Lys Asn
145 150 155 160

Ile Asn Tyr Glu Gly Ile Gln Met Ile Val Ser Gly Phe Gly Arg Thr
165 170 175

Asp Asp Leu Trp Asn Gly Gly Ala Ala Ser Glu Ile Leu Leu Trp Val
180 185 190

Tyr Gln Arg Gly Val Ser Asn Glu Glu Cys Leu Arg Trp Tyr Pro Thr
195 200 205

Ser Gln Val Ile Lys Glu Gln Thr Ile Cys Ala Gly Tyr Trp Asp Asn
210 215 220

Pro Ser Gln Ser Ser Cys Gln Gly Asp Ser Gly Gly Pro Leu Thr Ile
225 230 235 240

```
Ile Asp Ala Asp Gly Glu Arg Thr Gln Val Gly Ile Val Ser Ser Asp
                245                     250                 255

Pro Leu Leu Asp Ala Thr Val His Ser Pro Arg Val Thr Ser Pro Gly
            260                 265                 270

His Tyr His Asp Gly His Arg Gly Asp Arg His Gln Leu Arg Leu Gly
        275                 280                 285

Gln Arg Arg His Tyr Pro Asp Ser Ser Glu Ser Ser Leu Arg Ala Ala
    290                 295                 300

Ile Leu Pro Leu Glu Ser Ser Arg Ala Phe Ile Arg Arg Asn Gln Ser
305                 310                 315                 320

Ser Phe Arg Gly Gly Leu Cys Gln Pro Pro Arg Phe Pro Thr Arg Thr
                325                 330                 335

Val Pro Thr His Leu Pro Arg Arg Thr Leu Ala Ala Pro Pro Ser Glu
            340                 345                 350

Glu Glu Glu Ala Pro Glu Lys Val Arg Val Val Glu Tyr
        355                 360                 365
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<210> 55
<211> 36
<212> PRT
<213> H. punctigera

<400> 55

Ile Val Gly Gly Ser Leu Ser Ser Val Gly Gln Ile Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Asp Leu Ala Gly Gly Gln Ala Val Cys Gly Gly Ser
20 25 30

Leu Ile Ser Ala
35

<210> 56
<211> 30
<212> PRT
<213> H. punctigera

<400> 56

Ile Val Gly Gly Ser Thr Ser Ser Val Gly Gln Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ala Ser Phe Ala Gly Gly Gln Ala Val Cys Gly
20 25 30

<210> 57
<211> 37
<212> PRT
<213> H. punctigera

<400> 57

Ile Val Gly Gly Ser Ile Thr Asp Ile Ala Asn Val Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val Cys Gly Ala Ser
20 25 30

Leu Ile Ser His Asn
35

<210> 58
<211> 37
<212> PRT
<213> H. punctigera

<400> 58

Ile Val Gly Gly Ser Ile Thr Asn Ile Ala Asn Val Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val Cys Gly Ala Ser
20 25 30

Leu Ile Ser His Asn
35

<210> 59
<211> 37
<212> PRT
<213> H. punctigera

<400> 59

Ile Val Gly Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala
1   5   10   15

Gly Leu Leu Ala Ser Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser
            20   25   30

Leu Leu Asn Val Arg
        35

<210> 60
<211> 37
<212> PRT
<213> H. punctigera

<400> 60

Ile Val Gly Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala
1   5   10   15

Gly Leu Leu Ala Asn Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser
            20   25   30

Leu Leu Asn Gln Arg
        35

<210> 61
<211> 37
<212> PRT
<213> H. punctigera

<400> 61

Ile Val Gly Gly Ser Ser Ala Ser Leu Gly Gln Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Ser Leu Ile Tyr Ser Gly Gln Ser Val Cys Gly Gly Ser Leu
20 25 30

Leu Asn Gln Arg Arg
35

<210> 62
<211> 37
<212> PRT
<213> H. punctigera

<400> 62

Ile Val Ala Gly Trp Pro Ala Val Glu Gly Gln Ile Pro Tyr Gln Gly
1 5 10 15

Ser Leu Arg Met Val Ser Ala Ile Gly Gly Val Ser Ser Cys Gly Cys
20 25 30

Ser Leu Ile His Asn
35

<210> 63
<211> 235
<212> PRT
<213> H. punctigera

<400> 63

Ile Val Gly Gly Ser Ile Thr Asp Ile Ala Asn Val Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val Cys Gly Ala Ser
20 25 30

Leu Ile Ser His Asn Arg Leu Val Thr Ala Ala His Cys Lys Ser Asp
35 40 45

Gly Val Leu Thr Ala Asn Ser Phe Thr Val Val Leu Gly Ser Asn Thr
50 55 60

Leu Phe Phe Gly Gly Thr Arg Ile Asn Thr Asn Asp Val Val Met His
65 70 75 80

Pro Asn Trp Asn Pro Ser Thr Ala Ala Asn Asp Ile Ala Val Met Arg
85 90 95

Ile Ser Ser Val Ser Phe Ser Asn Val Ile Gln Pro Ile Ala Leu Pro
100 105 110

Ser Gly Asp Glu Leu Asn Asn Leu Phe Val Gly Ala Asn Ala Leu Ala
115 120 125

Phe Gly Phe Gly Arg Thr Ser Asp Gly Gly Ser Ile Gly Ser Asn Gln
130 135 140

Gln Val Ser Ser Val Thr Ile Pro Val Ile Thr Asn Asp Glu Cys Ala
145 150 155 160

Ala Val Tyr Gly Ser Ala Phe Val His Ser Ser Asn Ile Cys Thr Ser
165 170 175

Gly Ala Gly Gly Lys Gly Thr Cys Asn Gly Asp Ser Gly Gly Pro Leu
180 185 190

Ala Ile Asp Ser Asn Asn Glu Lys Ile Leu Ile Gly Val Thr Ser Tyr
195 200 205

Gly Ala Gln Ala Gly Cys Ala Ala Gly Leu Pro Ala Ala Phe Ala Arg
210 215 220

Val Thr Ser Phe Val Ser Trp Val Gln Ser Gln
225 230 235

<210> 64
<211> 235
<212> PRT
<213> H. punctigera

<400> 64

Ile Val Gly Gly Ser Ile Thr Asn Ile Ala Asn Val Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Thr Ile Phe Ile Phe Gln Ser Val Cys Gly Ala Ser
20 25 30

Leu Ile Ser His Asn Arg Leu Val Thr Ala Ala His Cys Lys Phe Asp
35 40 45

Gly Val Met Thr Ala Asn Ser Phe Thr Val Val Leu Gly Ser Asn Thr
50 55 60

Leu Phe Phe Gly Gly Thr Arg Ile Asn Thr Asn Asp Val Val Met His
65 70 75 80

Pro Asn Trp Asn Pro Ser Thr Val Ala Asn Asp Ile Ala Val Ile Arg
85 90 95

Ile Ser Ser Ile Val Tyr Asn Asn Val Ile Gln Pro Ile Ala Leu Pro
100 105 110

Ser Gly Asp Glu Leu Asp Asn Leu Phe Val Gly Ala Asn Ala Leu Ala
115 120 125

Ser Gly Phe Gly Arg Thr Ser Asp Ser Gly Gly Ile Gly Thr Asn Gln
130 135 140

Gln Leu Ser Ser Val Thr Ile Pro Val Ile Thr Asn Ala Glu Cys Ala
145 150 155 160

Ala Val Tyr Gly Pro Ala Phe Val His Asp Thr Asn Ile Cys Thr Ser
165 170 175

Gly Ala Gly Gly Lys Gly Thr Cys Asn Gly Asp Ser Gly Gly Pro Leu
180 185 190

Ala Val Asp Ser Asn Asp Lys Lys Ile Leu Ile Gly Val Thr Ser Tyr
195 200 205

Gly Ala Ala Asp Gly Cys Ala Ala Gly Phe Pro Ala Ala Phe Ala Arg
210 215 220

Val Thr Ser Phe Val Ser Trp Val Gln Ser Gln
225 230 235

<210> 65
<211> 234
<212> PRT
<213> H. punctigera

<400> 65

Ile Val Gly Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ala Ser Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser
20 25 30

Leu Leu Asn Val Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Arg Asn Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg
50 55 60

Leu Tyr Ser Gly Gly Thr Arg Leu Asn Thr Ala Ser Val Val Met His
65 70 75 80

Gly Ser Trp Asn Pro Asn Leu Val Arg Asn Asp Ile Ala Met Ile Asn
85 90 95

Leu Pro Ser Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu
100 105 110

Pro Ser Gly Asn Glu Leu Asn Asn Gln Phe Ala Gly Ala Thr Ala Thr
115 120 125

Ala Ser Gly Phe Gly Leu Ala Arg Asp Gly Gly Val Ile Asp Gly Asn
130 135 140

Leu Arg His Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Ser Gln
145 150 155 160

Ser Phe Pro Gly Leu Ile Gln Ala Ser Asn Val Cys Thr Ser Gly Ala
165 170 175

Asn Gly Arg Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val
180 185 190

Asn Ser Asn Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser
195 200 205

Ala Arg Gly Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Ser
210 215 220

Ser Tyr Ile Ser Trp Ile Asn Gln Arg Leu
225 230

<210> 66
<211> 234
<212> PRT
<213> H. punctigera

<400> 66

Ile Val Gly Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ala Asn Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser
20 25 30

Leu Leu Asn Gln Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Arg Asn Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg
50 55 60

Leu Phe Ser Gly Gly Thr Arg Leu Asp Thr Ala Ser Val Val Met His
65 70 75 80

Gly Ser Trp Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Met Ile Asn
85 90 95

Leu Pro Ser Asn Val Ala Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu
100 105 110

Pro Ser Gly Asn Glu Leu Asn Asn Asn Phe Asn Gly Ala Thr Ala Thr
115 120 125

Ala Ser Gly Phe Gly Leu Ala Arg Asp Gly Gly Ser Val Asp Gly Asn
130 135 140

Leu Arg His Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys Thr Val
145 150 155 160

Ser Phe Pro Gly Ile Ile Gln Ser Ser Asn Ile Cys Thr Ser Gly Ala
165 170 175

Asn Gly Arg Ser Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val
180 185 190

Asn Ser Asn Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser
195 200 205

Ala Arg Gly Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg Val Thr
210 215 220

Ser Phe Ile Ser Trp Ile Asn Gln Arg Leu
225 230

<210> 67
<211> 282
<212> PRT
<213> H. punctigera

<400> 67

Ile Val Gly Gly Ser Ser Ala Ser Leu Gly Gln Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Ser Leu Ile Tyr Ser Gly Gln Ser Val Cys Gly Gly Ser Leu
20 25 30

Leu Asn Gln Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp Gly
35 40 45

Ile Val Ala Gly Trp Pro Ala Val Glu Gly Gln Ile Pro Tyr Gln Gly
50 55 60

Ser Leu Arg Met Val Ser Ala Ile Gly Gly Val Ser Ser Cys Gly Cys
65 70 75 80

Ser Leu Ile His Asn Lys Trp Val Leu Thr Ala Ala His Cys Leu Ala
85 90 95

Asn Arg Asn Gln Ala Thr Ser Leu Thr Val Ile Leu Gly Ser Ile Asn
100 105 110

```
Leu Phe Phe Gly Gly Thr Arg Leu Asn Ser Asn Ser Val Val Met His
        115                 120                 125

Gly Ser Trp Asn Pro Asn Leu Ile Arg Asn Asp Ile Ala Ile Ile Asn
    130                 135                 140

Leu Pro Ser Asn Val Gly Thr Ser Gly Asn Ile Ala Pro Ile Ala Leu
145                 150                 155                 160

Pro Ser Gly Asn Glu Leu Asn Asn Gln Phe Ala Gly Phe Thr Ala Thr
                165                 170                 175

Ala Ser Gly Phe Gly Leu Thr Arg Asp Gly Gly Asn Val Ser Pro Thr
            180                 185                 190

Leu Asn His Val Asn Leu Pro Val Ile Thr Asn Asn Val Cys Trp Gln
        195                 200                 205

Ser Phe Pro Leu Tyr Ile Gln Ser Thr Asn Ile Cys Thr Ser Gly Ala
    210                 215                 220

Asn Gly Arg Gly Thr Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Val
225                 230                 235                 240

Thr Ser Asn Asn Arg Arg Ile Leu Ile Gly Val Thr Ser Phe Gly Ser
                245                 250                 255
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Asp Arg Gly Cys Gln Val Gly Ala Pro Ala Ala Phe Ala Arg Val Thr
260 265 270

Ser Tyr Ile Ser Trp Ile Asn Gln Arg Leu
275 280

<210> 68
<211> 256
<212> PRT
<213> H. punctigera

<400> 68

Ile Val Ala Gly Trp Pro Ala Val Glu Gly Gln Ile Pro Tyr Gln Gly
1 5 10 15

Ser Leu Arg Met Val Ser Ala Ile Gly Gly Val Ser Ser Cys Gly Cys
20 25 30

Ser Leu Ile His Asn Lys Trp Val Leu Thr Ala Ala His Cys Leu Ala
35 40 45

Asn Arg Ile Thr Phe Val Val Arg Phe Gly Leu Thr Asn Leu Thr Arg
50 55 60

Pro Glu Ile Leu Val Glu Ser Thr Asn Lys Tyr Ile His Pro Glu Tyr
65 70 75 80

Asp Glu Ile Arg Ala Gly Val Gln Thr Ala Asp Leu Ala Leu Val Gly
85 90 95

Leu Asp His Glu Ile Glu Tyr Ser Ala Asn Val Gln Pro Ser Arg Leu
100 105 110

Met Ser Ser Ala Gln Lys Asn Ile Asn Tyr Glu Gly Ile Gln Met Ile
115 120 125

Val Ser Gly Phe Gly Arg Thr Asp Asp Leu Trp Asn Gly Gly Ala Ala
130 135 140

Ser Glu Ile Leu Leu Trp Val Tyr Gln Arg Gly Val Ser Asn Glu Glu
145 150 155 160

Cys Leu Arg Trp Tyr Pro Thr Ser Gln Val Ile Lys Glu Gln Thr Ile
165 170 175

Cys Ala Gly Tyr Trp Asp Asn Pro Ser Gln Ser Ser Cys Gln Gly Asp
180 185 190

Ser Gly Gly Pro Leu Thr Ile Ile Asp Ala Asp Gly Glu Arg Thr Gln
195 200 205

Ser Arg Tyr Cys Glu Leu Arg Ile His Cys Trp Asn Ala Thr Ala His
210 215 220

Ser Pro Gln Gly Tyr Val Arg Pro Gly His Tyr His Asp Trp Phe Thr
225 230 235 240

Glu Val Thr Gly Ile Asn Phe Asp Trp Asp Ser Asp Ala Ile Ile Pro
245 250 255

<210> 69
<211> 236
<212> PRT
<213> H. punctigera

<400> 69

Ile Val Gly Gly Ser Leu Ser Ser Val Gly Gln Ile Pro Tyr Gln Ala
1 5 10 15

Gly Leu Val Ile Asp Leu Ala Gly Gly Gln Ala Val Cys Gly Gly Ser
20 25 30

Leu Ile Ser Ala Ser Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Gln Asn Gln Ala Trp Arg Phe Thr Val Val Leu Gly Ser Thr Thr
50 55 60

Leu Phe Ser Gly Gly Thr Arg Ile Pro Thr Ser Asn Val Val Met His
65 70 75 80

Gly Ser Trp Thr Pro Ser Leu Ile Arg Asn Asp Val Ala Val Ile Arg
85 90 95

Leu Gly Thr Asn Val Ala Thr Ser Asn Thr Ile Ala Ile Ile Ala Leu
100 105 110

Pro Ser Gly Ser Gln Ile Asn Glu Asn Phe Ala Gly Glu Thr Ala Leu
115 120 125

Ala Ser Gly Phe Gly Leu Thr Ser Asp Thr Gly Ser Ile Ser Ser Asn
130 135 140

Gln Ala Leu Ser His Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys
145 150 155 160

Arg Asn Ser Phe Pro Leu Leu Ile Gln Asp Ser Asn Ile Cys Thr Ser
165 170 175

Gly Ala Asn Gly Arg Ser Thr Cys Arg Gly Asp Ser Gly Gly Pro Leu
180 185 190

Val Val Thr Arg Asn Asn Arg Pro Leu Leu Ile Gly Ile Thr Ser Phe
195 200 205

Gly Ser Ala Arg Gly Cys Gln Val Gly Ser Pro Ala Ala Phe Ala Arg
210 215 220

Val Thr Ser Tyr Ile Ser Trp Ile Asn Gly Gln Leu
225 230 235

<210> 70
<211> 224
<212> PRT
<213> H. punctigera

<400> 70

Ile Val Gly Gly Tyr Thr Cys Glu Glu Asn Ser Leu Pro Tyr Gln Val
1 5 10 15

Ser Leu Asn Ser Gly Ser His Phe Cys Gly Gly Ser Leu Ile Ser Glu
20 25 30

Gln Trp Val Val Ser Ala Ala His Cys Tyr Lys Thr Arg Ile Gln Val
35 40 45

Arg Leu Gly Glu His Asn Ile Lys Val Leu Glu Gly Asn Glu Gln Phe
50 55 60

Ile Asn Ala Ala Lys Ile Ile Arg His Pro Lys Tyr Asn Arg Asp Thr
65 70 75 80

Leu Asp Asn Asp Ile Met Leu Ile Lys Leu Ser Ser Pro Ala Val Ile
85 90 95

Asn Ala Arg Val Ser Thr Ile Ser Leu Pro Thr Ala Pro Pro Ala Ala
100 105 110

Gly Thr Glu Cys Leu Ile Ser Gly Trp Gly Asn Thr Leu Ser Phe Gly
115 120 125

Ala Asp Tyr Pro Asp Glu Leu Lys Cys Leu Asp Ala Pro Val Leu Thr
130 135 140

Gln Ala Glu Cys Lys Ala Ser Tyr Pro Gly Lys Ile Thr Asn Ser Met
145 150 155 160

Phe Cys Val Gly Phe Leu Glu Gly Gly Lys Asp Ser Cys Gln Arg Asp
165 170 175

Ser Gly Gly Pro Val Val Cys Asn Gly Gln Leu Gln Gly Val Val Ser
180 185 190

Trp Gly His Gly Cys Ala Trp Lys Asn Arg Pro Gly Val Tyr Thr Lys
195 200 205

Val Tyr Asn Tyr Val Asp Trp Ile Lys Asp Thr Ile Ala Ala Asn Ser
210 215 220

<210> 71
<211> 275
<212> PRT
<213> H. armigera

<400> 71

Val His Leu Glu Asp Ser Ile Asp Leu Glu Asp Ile Thr Ala Trp Gly
1 5 10 15

Tyr Leu Thr Lys Phe Gly Ile Pro Glu Ala Glu Lys Ile Arg Asn Ala
20 25 30

Glu Glu Ala Ser Ser Ala Ser Arg Ile Val Gly Gly Ser Leu Ser Ser
35 40 45

Leu Gly Gln Ile Pro Tyr Gln Ala Gly Leu Val Ile Asp Leu Ser Gly
50 55 60

Gly Gln Ala Val Cys Gly Gly Ser Leu Ile Ser Ala Ser Arg Val Leu
65 70 75 80

Thr Ala Ala His Cys Trp Phe Asp Gly Gln Asn Gln Ala Trp Arg Phe
85 90 95

Thr Val Val Leu Gly Ser Thr Thr Leu Phe Ser Gly Gly Thr Arg Ile
100 105 110

Ala Thr Ser Asn Val Val Met His Gly Ser Trp Thr Pro Ser Leu Ile
115 120 125

Arg Asn Asp Val Ala Val Ile Arg Leu Gly Thr Asn Val Gly Thr Ser
130 135 140

Asn Thr Ile Ala Ile Ile Ala Leu Pro Ser Gly Ser Gln Ile Asn Glu
145 150 155 160

Asn Phe Ala Gly Glu Thr Ala Leu Ala Ser Gly Phe Gly Leu Thr Ser
165 170 175

Asp Ser Gly Ser Ile Ser Ser Asn Gln Ala Leu Ser His Val Asn Leu
180 185 190

Pro Val Ile Thr Asn Ala Val Cys Arg Ser Ser Phe Pro Leu Leu Ile
195 200 205

Gln Asp Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg Ser Thr Cys
210 215 220

Arg Gly Asp Ser Gly Gly Pro Leu Val Val Thr Arg Asn Ser Arg Pro
225 230 235 240

Leu Leu Ile Gly Ile Thr Ser Phe Gly Ser Ala Arg Gly Cys Gln Val
245 250 255

Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Tyr Ile Ser Trp Ile
260 265 270

Asn Gly Gln
275

<210> 72
<211> 275
<212> PRT
<213> H. punctigera

<400> 72

Val His Leu Glu Asp Ser Ile Asp Leu Glu Asp Ile Thr Ala Trp Gly
1 5 10 15

Tyr Leu Thr Lys Phe Gly Ile Pro Glu Ala Glu Lys Ile Arg Asn Ala
20 25 30

Glu Glu Ala Ser Ser Ala Ser Arg Ile Val Gly Gly Ser Leu Ser Ser
35 40 45

Leu Gly Gln Ile Pro Tyr Gln Ala Gly Leu Val Ile Asp Leu Ala Gly
50 55 60

Gly Gln Ala Val Cys Gly Gly Ser Leu Ile Ser Ala Ser Arg Val Leu
65 70 75 80

Thr Ala Ala His Cys Trp Phe Asp Gly Gln Asn Gln Ala Trp Arg Phe
85 90 95

Thr Val Val Leu Gly Ser Thr Thr Leu Phe Ser Gly Gly Thr Arg Ile
100 105 110

Pro Thr Ser Asn Val Val Met His Gly Ser Trp Thr Pro Ser Leu Ile
115 120 125

Arg Asn Asp Val Ala Val Ile Arg Leu Gly Thr Asn Val Gly Thr Ser
130 135 140

Asn Thr Ile Ala Ile Ile Ala Leu Pro Ser Gly Ser Gln Ile Asn Glu
145 150 155 160

Asn Phe Ala Gly Glu Thr Ala Leu Ala Ser Gly Phe Gly Leu Thr Ser
165 170 175

Asp Thr Gly Ser Ile Ser Ser Asn Gln Ala Leu Ser His Val Asn Leu
180 185 190

Pro Val Ile Thr Asn Ala Val Cys Arg Asn Ser Phe Pro Leu Leu Ile
195 200 205

Gln Asp Ser Asn Ile Cys Thr Ser Gly Ala Asn Gly Arg Ser Thr Cys
210 215 220

Arg Gly Asp Ser Gly Gly Pro Leu Val Val Thr Arg Asn Asn Arg Pro
225 230 235 240

Leu Leu Ile Gly Ile Thr Ser Phe Gly Ser Ala Arg Gly Cys Gln Val
245 250 255

Gly Ser Pro Ala Ala Phe Ala Arg Val Thr Ser Tyr Ile Ser Trp Ile
260 265 270

Asn Gly Gln
275

<210> 73
<211> 230
<212> PRT
<213> H. punctigera

<400> 73

Ile Val Asn Gly Glu Asp Ala Val Pro Gly Ser Trp Pro Trp Gln Val
1   5   10   15

Ser Leu Gln Asp Ser Thr Gly Phe His Phe Cys Gly Gly Ser Leu Ile
            20   25   30

Ser Glu Asp Trp Val Val Thr Ala Ala His Cys Gly Val Thr Thr Ser
        35   40   45

Asp Val Val Val Ala Gly Glu Phe Asp Gln Gly Ser Ser Ser Glu Lys
    50   55   60

Ile Gln Lys Leu Lys Ile Ala Lys Val Phe Lys Asn Ser Lys Tyr Asn
65   70   75   80

Ser Leu Thr Ile Asn Asn Asp Ile Thr Leu Leu Lys Leu Ala Thr Pro
                85   90   95

Ala Gln Phe Ser Glu Thr Val Ser Ala Val Cys Leu Pro Ser Ala Asp
            100   105   110

Glu Asp Phe Pro Ala Gly Met Leu Cys Ala Thr Thr Gly Trp Gly Lys
        115   120   125

Thr Lys Tyr Asn Ala Leu Lys Thr Pro Asp Lys Leu Gln Gln Ala Thr
130 135 140

Leu Pro Ile Val Ser Asn Thr Asp Cys Arg Lys Tyr Trp Gly Ser Arg
145 150 155 160

Val Thr Asp Val Met Ile Cys Ala Gly Ala Ser Gly Val Ser Ser Cys
165 170 175

Met Gly Asp Ser Gly Gly Pro Leu Val Cys Gln Lys Asn Gly Ala Trp
180 185 190

Thr Leu Ala Gly Ile Val Ser Trp Gly Ser Ser Thr Cys Ser Thr Ser
195 200 205

Thr Pro Ala Val Tyr Ala Arg Val Thr Ala Leu Met Pro Trp Val Gln
210 215 220

Glu Thr Leu Ala Ala Asn
225 230

<210> 74
<211> 230
<212> PRT
<213> H. punctigera

<400> 74

Ile Val Asn Gly Glu Glu Ala Val Pro Gly Ser Trp Pro Trp Gln Val
1 5 10 15

Ser Leu Gln Asp Lys Thr Gly Phe His Phe Cys Gly Gly Ser Leu Ile
20 25 30

Asn Glu Asn Trp Val Val Thr Ala Ala His Cys Gly Val Thr Thr Ser
35 40 45

Asp Val Val Val Ala Gly Glu Phe Asp Gln Gly Leu Glu Thr Glu Asp
50 55 60

Thr Gln Val Leu Lys Ile Gly Lys Val Phe Lys Asn Pro Lys Phe Ser
65 70 75 80

Ile Leu Thr Val Arg Asn Asp Ile Thr Leu Leu Lys Leu Ser Thr Ala
85 90 95

Ala Ser Phe Ser Gln Thr Val Ser Ala Val Cys Leu Pro Ser Ala Ser
100 105 110

Asp Asp Phe Ala Ala Gly Thr Thr Cys Val Thr Thr Gly Trp Gly Leu
115 120 125

Thr Arg Tyr Thr Asn Ala Asn Thr Pro Asp Arg Leu Gln Gln Ala Ser
130 135 140

Leu Pro Leu Leu Ser Asn Thr Asn Cys Lys Lys Tyr Trp Gly Thr Lys
145 150 155 160

Ile Lys Asp Ala Met Ile Cys Ala Gly Ala Ser Gly Val Ser Ser Cys
165 170 175

Met Gly Asp Ser Gly Gly Pro Leu Val Cys Lys Gln Asn Gly Ala Trp
180 185 190

Thr Leu Val Gly Ile Val Ser Trp Gly Ser Ser Thr Cys Ser Thr Ser
195 200 205

Thr Pro Gly Val Tyr Ala Arg Val Thr Ala Leu Val Asn Trp Val Gln
210 215 220

Gln Thr Leu Ala Ala Asn
225 230

<210> 75
<211> 237
<212> PRT
<213> H. punctigera

<400> 75

Ile Val Gly Gly Ser Thr Ser Ser Leu Gly Ala Phe Pro Tyr Gln Ala
1 5 10 15

Gly Leu Leu Ala Ser Phe Ala Ser Gly Gln Gly Val Cys Gly Gly Ser
20 25 30

Leu Leu Asn Val Arg Arg Val Leu Thr Ala Ala His Cys Trp Phe Asp
35 40 45

Gly Arg Asn Gln Ala Arg Ser Phe Thr Val Val Leu Gly Ser Val Arg
50 55 60

Leu Tyr Ser Gly Gly Thr Arg Leu Asn Thr Ala Ser Val Val Met His
65 70 75 80

Gly Ser Trp Asn Pro Asn Leu Val Arg Thr Ile Asn Asn Asp Ile Ala
85 90 95

Met Ile Asn Leu Pro Ser Asn Val Ala Thr Ser Gly Asn Ile Ala Pro
100 105 110

Ile Ala Leu Pro Ser Gly Asn Glu Leu Asn Asn Gln Phe Ala Gly Ala
115 120 125

Thr Ala Thr Ala Ser Gly Phe Gly Leu Ala Arg Asp Gly Gly Val Ile
130 135 140

Asp Gly Asn Leu Arg His Val Asn Leu Pro Val Ile Thr Asn Ala Val
145 150 155 160

Cys Ser Gln Ser Phe Pro Gly Leu Ile Gln Ala Ser Asn Val Cys Thr
165 170 175

Ser Gly Ala Asn Gly Arg Ser Thr Cys Gln Gly Gly Asp Ser Gly Gly
180 185 190

Pro Leu Val Asn Ser Asn Asn Arg Arg Ile Leu Ile Gly Val Thr Ser
195 200 205

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<400> 76

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Gly Leu Val Ile Asp Leu Ala Gly Gly Gln Ala Val Cys Gly Gly Ser
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35 40 45

Gly Gln Asn Gln Ala Trp Arg Phe Thr Val Val Leu Gly Ser Thr Thr
50 55 60

Leu Phe Ser Gly Gly Thr Arg Leu Asn Ile Pro Ser Ser Asn Met His
65 70 75 80

Gly Ser Trp Asn Pro Ser Leu Ile Arg Asn Asp Val Ala Val Ile Arg
85 90 95

Leu Gly Thr Asn Val Ala Thr Ser Asn Thr Ile Ala Ile Ile Ala Leu
100 105 110

Pro Ser Gly Ser Gln Ile Asn Glu Asn Phe Ala Gly Glu Thr Ala Leu
115 120 125

Ala Ser Gly Phe Gly Leu Thr Ser Tyr Thr Gly Ser Ile Ser Ser Asn
130 135 140

Gln Ala Leu Ser His Val Asn Leu Pro Val Ile Thr Asn Ala Val Cys
145 150 155 160

Arg Asn Ser Phe Ser Leu Leu Ile Gln Asp Ser Asn Ile Cys Thr Ser
165 170 175

Gly Ala Asn Gly Arg Ser Thr Cys Arg Gly Asp Ser Gly Gly Pro Leu
180 185 190

Val Val Thr Arg Asn Asn Arg Pro Leu Leu Ile Gly Val Thr Ser Phe
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Val Thr Ser Tyr Ile Ser Trp Ile Asn Gly Gln Leu
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Ile Glu Leu Leu Lys Glu Phe Glu Cys Asn Gly Lys Gln Phe Trp Pro
35 40 45

Glu Leu Ile Gly Val Pro Thr Lys Leu Ala Lys Glu Ile Ile Glu Lys
50 55 60

Glu Asn Ser Leu Ile Asn Asn Val Gln Ile Leu Leu Asn Gly Ser Pro
65 70 75 80

Val Thr Met Asp Tyr Arg Cys Asn Arg Val Arg Leu Phe Asp Asn Ile
85 90 95

Leu Gly Ser Val Val Gln Ile Pro Arg Val Ala
100 105

<210> 78
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<212> PRT
<213> peptide

<400> 78

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Ser Phe Glu Thr Leu Leu Ala Arg Lys Glu Ser Asp Gly Pro Glu Val
20 25 30

Ile Glu Leu Leu Lys Glu Phe Glu Cys Asn Gly Lys Gln Phe Trp Pro
35 40 45

Glu Leu Ile Gly Val Pro Thr Lys Leu Ala Lys Glu Ile Ile Glu Lys
50 55 60

Glu Asn Ser Leu Ile Asn Asn Val Gln Ile Leu Leu Asn Gly Ser Pro
65 70 75 80

Val Ala Met Asp Tyr Arg Cys Asn Arg Val Arg Leu Phe Asp Asn Ile
85 90 95

Leu Gly Ser Val Val Gln Ile Pro Arg Val Ala
100 105

<210> 79
<211> 71
<212> PRT
<213> peptide

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1 5 10 15

Val Pro Thr Lys Leu Ala Lys Glu Ile Ile Glu Lys Gln Asn Ser Leu
20 25 30

Ile Ser Asn Val His Ile Leu Leu Asn Gly Ser Pro Val Thr Met Asp
35 40 45

Phe Arg Cys Asn Arg Val Arg Leu Phe Asp Asp Ile Leu Gly Ser Val
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Val Gln Ile Pro Arg Val Ala
65 70

<210> 80
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<212> PRT
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20 25 30

Ile Glu Leu Gln Lys Glu Phe Glu Cys Asn Gly Lys Gln Arg Trp Pro
35 40 45

Glu Leu Ile Gly Val Pro Thr Lys Leu Ala Lys Gly Ile Ile Glu Lys
50 55 60

Glu Asn Ser Leu Ile Thr Asn Val Gln Ile Leu Leu Asn Gly Ser Pro
65 70 75 80

Val Thr Met Asp Tyr Arg Ser Asn Arg Val Arg Leu Phe Asp Asn Ile
85 90 95

Leu Gly Asp Val Val Gln Ile Pro Arg Val
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<212> PRT
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20 25 30

Ile Lys Leu Leu Lys Glu Ser Glu Ser Glu Ser Trp Cys Lys Gly Lys
35 40 45

Gln Phe Trp Pro Glu Leu Ile Gly Val Pro Thr Lys Leu Ala Lys Glu
50 55 60

Ile Ile Glu Lys Glu Asn Pro Ser Ile Asn Asp Val Pro Ile Ile Leu
65 70 75 80

Asn Gly Thr Pro Val Pro Ala Asp Phe Arg Cys Asn Arg Val Arg Leu
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<210> 82
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Ile Glu Leu Leu Lys Glu Phe Asp Ser Asn Leu Met Cys Glu Gly Lys
35 40 45

Gln Met Trp Pro Glu Leu Ile Gly Val Pro Thr Lys Leu Ala Lys Glu
50 55 60

Ile Ile Glu Lys Glu Asn Pro Ser Ile Thr Asn Ile Pro Ile Leu Leu
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<210> 83
<211> 107
<212> PRT
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<400> 83

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Asp Val Ser Gln Ser Gly Cys Pro Gly Val Thr Lys Glu Arg Trp Pro
35 40 45

Glu Leu Leu Gly Thr Pro Ala Lys Phe Ala Met Gln Ile Ile Gln Lys
50 55 60

Glu Asn Pro Lys Leu Thr Asn Val Gln Thr Ile Leu Asn Gly Gly Pro
65 70 75 80

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35 40 45

Val Val Thr Ala Asp Tyr Arg Pro Asn Arg Val Arg Ile Phe Val Asp
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Ile Val Ala Gln Thr Pro His Ile Gly
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Thr Val Asp Gln Ala Arg Glu Tyr Phe Thr Leu His Tyr Pro Gln Tyr
20 25 30

Asp Val Tyr Phe Leu Pro Glu Gly Ser Pro Val Thr Leu Asp Leu Arg
35 40 45

Tyr Asn Arg Val Arg Val Phe Tyr Asn Pro Gly Thr Asn Val Val Asn
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His Val Pro His Val Gly
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<210> 88
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<210> 90
<211> 864
<212> DNA
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<400> 90
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tccctgatca gcgcttcccg cgtactgacc gctgctcact gctggttcga cggccaaaac 300

caggcctgga gattcaccgt tgttcttggt tccaccacct tgttctctgg cggtaccaga 360

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ctgccagtga tcaccaacgc tgtgtgcaga aattcattcc ccctgctgat ccaggactct 660

aacatttgca ccagcggtgc caacggcagg agcacttgcc gcggtgactc cggcggtcct 720

ctcgtcgtca ccaggaacaa cagaccactc ttgatcggta tcacctcttt cggatctgcc 780

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<400> 91

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20 25 30

Pro Glu Ala Glu Lys Ile Arg Asn Ala Glu Glu Ala Ser Ser Ala Ser
35 40 45

Arg Ile Val Gly Gly Ser Leu Ser Ser Val Gly Gln Ile Pro Tyr Gln
50 55 60

Ala Gly Leu Val Ile Asp Leu Ala Gly Gly Gln Ala Val Cys Gly Gly
65 70 75 80

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Ser Leu Ile Ser Ala Ser Arg Val Leu Thr Ala Ala His Cys Trp Phe
                85                  90                  95

Asp Gly Gln Asn Gln Ala Trp Arg Phe Thr Val Val Leu Gly Ser Thr
            100                 105                 110

Thr Leu Phe Ser Gly Gly Thr Arg Ile Pro Thr Ser Asn Val Val Met
        115                 120                 125

His Gly Ser Trp Thr Pro Ser Leu Ile Arg Asn Asp Val Ala Val Ile
    130                 135                 140

Arg Leu Gly Thr Asn Val Ala Thr Ser Asn Thr Ile Ala Ile Ile Ala
145                 150                 155                 160

Leu Pro Ser Gly Ser Gln Ile Asn Glu Asn Phe Ala Gly Glu Thr Ala
                165                 170                 175

Leu Ala Ser Gly Phe Gly Leu Thr Ser Asp Thr Gly Ser Ile Ser Ser
            180                 185                 190

Asn Gln Ala Leu Ser His Val Asn Leu Pro Val Ile Thr Asn Ala Val
        195                 200                 205

Cys Arg Asn Ser Phe Pro Leu Leu Ile Gln Asp Ser Asn Ile Cys Thr
    210                 215                 220

Ser Gly Ala Asn Gly Arg Ser Thr Cys Arg Gly Asp Ser Gly Gly Pro
225                 230                 235                 240
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Leu Val Val Thr Arg Asn Asn Arg Pro Leu Leu Ile Gly Ile Thr Ser
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Phe Gly Ser Ala Arg Gly Cys Gln Val Gly Ser Pro Ala Ala Phe Ala
260 265 270

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275 280 285

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<400> 92
gatcaacggc cagctctaaa agctt 25

<210> 93
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<400> 93

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1 5 10 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000524

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 9/76, 15/57, 15/12; A01H 5/00; A01N 63/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DGENE, SWISS PROT, EMBL, GENBANK, PIR: SEQ ID NO: 2, 3 and 5 (BLASTN, BLASTX)
WPIDS, MEDLINE, CA BIOSIS : chymotrypsin; sepharose; benzamidine; affinity chromatography; inhibitor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Heath, R. L. et al., 1997, Proteinase inhibitors from *Nicotiana alata* enhance plant resistance to insect pests, ***Journal of Insect Physiology***, 43:833-842	1-48
A	Bown, D. P. et al, 1997, Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, ***Helicoverpa armigera***, are members of complex multigene families, ***Insect Biochemistry and Molecular Biology***, 27:625-638.	1-48

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 2004	08 JUL 2004

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**TERRY MOORE** Telephone No : (02) 6283 2632

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000524

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Gatehouse, L. N. et al., 1997, Characterisation of major midgut proteinase cDNAs from Helicoverpa armigera larvae and changes in gene expression in response to four proteinase inhibitors in the diet, ***Insect Biochemistry and Molecular Biology***, 27:929-944.	1-48
A	Mazumdar-Leighton, S. and Broadway, R., 2001, Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor, ***Insect Biochemistry and Molecular Biology***, 31:633-644.	1-48
A	DE 3135541 A (Bayer AG) 24 March 1983 Abstract	46
A	Hjelmeland, K., and Raa, J., 1982, Characteristics of two trypsin type isozymes isolated from the arctic fish capelin (Mallotus villosus), ***Comparative Biochemistry and Physiology B***, 71:557-62. Abstract	46
A	Sakal, E.et al., 1989, Purification and characterization of trypsins from the digestive tract of Locusta migratoria, ***International Journal of Peptide and Protein Research***, 34:498-505 Abstract	46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000524

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **29-43, 47, 48 (partially)**
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   See Supplemental Box

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

Supplemental Box

Continuation of Boxes No II and III

Observations where certain claims were found unsearchable (Box II)

The scope of claims 29-43, 48 is so inadequately supported by the specification that a meaningful search covering the full scope of the claims could not be carried out. In particular, the claims do not define the matter for which protection is sought in terms of the technical features of the invention.

Claims 29-43 relate to an antagonist of a chymotrypsin HpCh5 from *Helicoverpa* spp, compositions comprising said antagonist and genetically modified plants that produce an antgonist of chymotrypsin HpCh5. With respect to antagonists of the chymotrypsin isolated from *Helicoverpa* it is considered that the invention extends to
(a) substances that are derivatives of the gene identified in the present application, for example antisense, and
(b) substances that are isolated in methods that necessarily utilise the protein and/or gene of the present invention.

No meaningful search can be performed on the full scope of the claims. As such, claims 29-43 have been searched in as far as they relate to derivatives of HpCh5 (eg antisense, antibodies) that antagonise its activity or expression.

Claim 47 has been searched with respect to the use of HpCh5 to screen for potential antagonists of its activity.

Claim 48 is directed to an inhibitor of chymotrypsin identified by a method of screening comprising contacting a NaPI-insensitive chymotrypsin with a potential antagonist and screening for chymotrypsin activity. This is not a claim to a derivative of HpCh5 or a compound produced using HpCh5, it is a claim that encompasses an independent compound that inherently antagnoises HpCHh5 and whose engineering or isolation owes nothing to the teachings of the patent application. Thus the claim may encompass known substances inherently possessing the stated properties. No meaningful search can be performed on the full scope of the claim. The claim has been searched in as far as it relates to a derivative of HpCh5 (eg antisense) that antagonises its activity or expression.

Observations where unity of invention is lacking (Box III)

The International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-45, 47, 48 directed to a chymotrypsin polypeptide from *Helicoverpa* sp. wherein said polypeptide exhibits resistance to a proteinase inhibitor from *Nicotiana alata*. The claims also relate to the nucleic acid molecule encoding said polypeptide, expression vectors and genetically modified cells comprising said nucleic acid molecules, and methods that use the chymotrypsin polypeptide from Helicoverpa sp.

   It is considered that the chymotrypsin polypeptide and the nucleic acid molecule encoding it represents a first "special technical feature".

2. Claim 46 relating to a method for the isolation of individual isoforms of chymotrypsin based on sequential steps of affinity chromatography using benzamidine sepharose, the proteinase inhibitor C1 and Pot I, Pot II or chymostatin.. It is considered that this method represents a second "special technical feature".

Since the abovementioned groups of claims do not share any of the technical features identified, a "technical relationship" between the inventions, as defined in PCT rule 13.2 does not exist. Accordingly the international application does not relate to one invention or to a single inventive concept.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/000524

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
DE	3135541	DK	399982	FR	2512445	JP	58055430

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

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